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# Optimization of anti-CD20 humanized antibody hu8E4 by site-directed mutation based on epitope analysis

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ABSTRACT

Despite the effectiveness of the anti-CD20 chimeric antibody (mAb), rituximab, in treating B-cell lymphomas, its efficacy remains variable and often modest. Hu8E4 is an anti-CD20 humanized antibody which exhibits markedly higher antitumor activity compared with rituximab. Previous studies have indicated that rituximab and almost all known anti-CD20 murine mAbs recognize the A170/P172 motif within the large extracellular loop of CD20. In this study, we demonstrated that hu8E4 also recognized the A170/P172 motif, suggesting that the epitopes recognized by rituximab and hu8E4 are very similar. Based on this, three single mutations (D57E, Y102K and Y102T) at the heavy chain variable region that can improve the affinity of rituximab were transferred to hu8E4. The results showed that D57E and Y102T but not Y102K successfully enhanced the binding of hu8E4 to CD20. Out of these hu8E4 mutants, hu8E4$_{D57E}$ exhibited the highest affinity. The in vitro and in vivo antitumor activity of hu8E4$_{D57E}$ was further investigated. Our data indicated that hu8E4$_{D57E}$ was as effective as hu8E4 in mediating CDC and inducing apoptosis in B-lymphoma cells, but it was more potent in ADCC than hu8E4. Importantly, hu8E4$_{D57E}$ was shown to be significantly more effective than Hu8E4 in prolonging the survival of SCID mice bearing disseminated B-lymphoma cells, suggesting that it might be a promising therapeutic agent for B-cell lymphomas. Moreover, this study also suggests that the mutations that can improve the affinity of rituximab may be transferred to other anti-CD20 murine mAbs to enhance their binding to CD20.


## 1. Introduction

The CD20 molecule is present on >90% of B-cell lymphomas and is neither shed nor internalized after antibody binding, making it an effective target for immunotherapeutic removal of malignant B cells [1–3]. The anti-CD20 chimeric antibody rituximab (IDEC-C2B8, Rituxan) is the first approved monoclonal antibody (mAb) drug for use in relapsed or refractory low-grade or follicular B-cell non-Hodgkin's lymphoma [4–6]. Previous studies have suggested that several mechanisms might be involved in providing therapeutic efficacy, including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and the induction of apoptosis [7,8]. The relative contributions of these different mechanisms of action are still a matter of debate.

Despite the effectiveness of rituximab, only 48% of patients respond to the treatment and complete responses are less than 10%. There is an urgent need to develop more effective CD20-targeting antibody agents for the treatment of B-cell lymphomas. Based on the crystal structure of rituximab Fab-CD20 epitope peptide complex we determined previously [9], a computational method was used to improve the binding avidity of rituximab to CD20 antigen [10]. Rituximab mutants with higher affinity than that of rituximab were obtained, and our data demonstrated that these mutants showed more potent antitumor activity [11].

Previous studies have demonstrated that rituximab and almost all known anti-CD20 murine mAbs, including 1F5, AT80, and 2H7, recognize the A170/P172 motif within the large extracellular loop of CD20 [12]. However, all of the anti-CD20

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fully human mAbs recognize a completely novel epitope located N-terminally of the A170/P172 motif, also including the small extracellular loop of CD20 [12]. Hu8E4, an anti-CD20 humanized antibody recently developed by our laboratory [13], is as effective as rituximab in mediating ADCC and inducing apoptosis in B-lymphoma cells, but it exhibits much more potent CDC than rituximab [13]. In this study, we demonstrated that hu8E4 also recognized the A170/P172 motif. Together, these data suggest that the epitopes recognized by rituximab and hu8E4 are very similar. Based on this, we asked if the mutations that improved the affinity of rituximab could be transferred to hu8E4 to enhance its binding to CD20, thus improving its antitumor activity. In the present study, three single mutations that can improve the affinity of rituximab were transferred to hu8E4. The resulting hu8E4 mutants were further investigated for their binding activity and antitumor effect.

## 2. Materials and methods

### 2.1. Cell lines, antibodies, and animals

The Chinese hamster ovary cell line CHO–K1 and two CD20-positive human B-cell lymphoma cell lines, Raji and Daudi, were obtained from the American Type Culture Collection (ATCC).

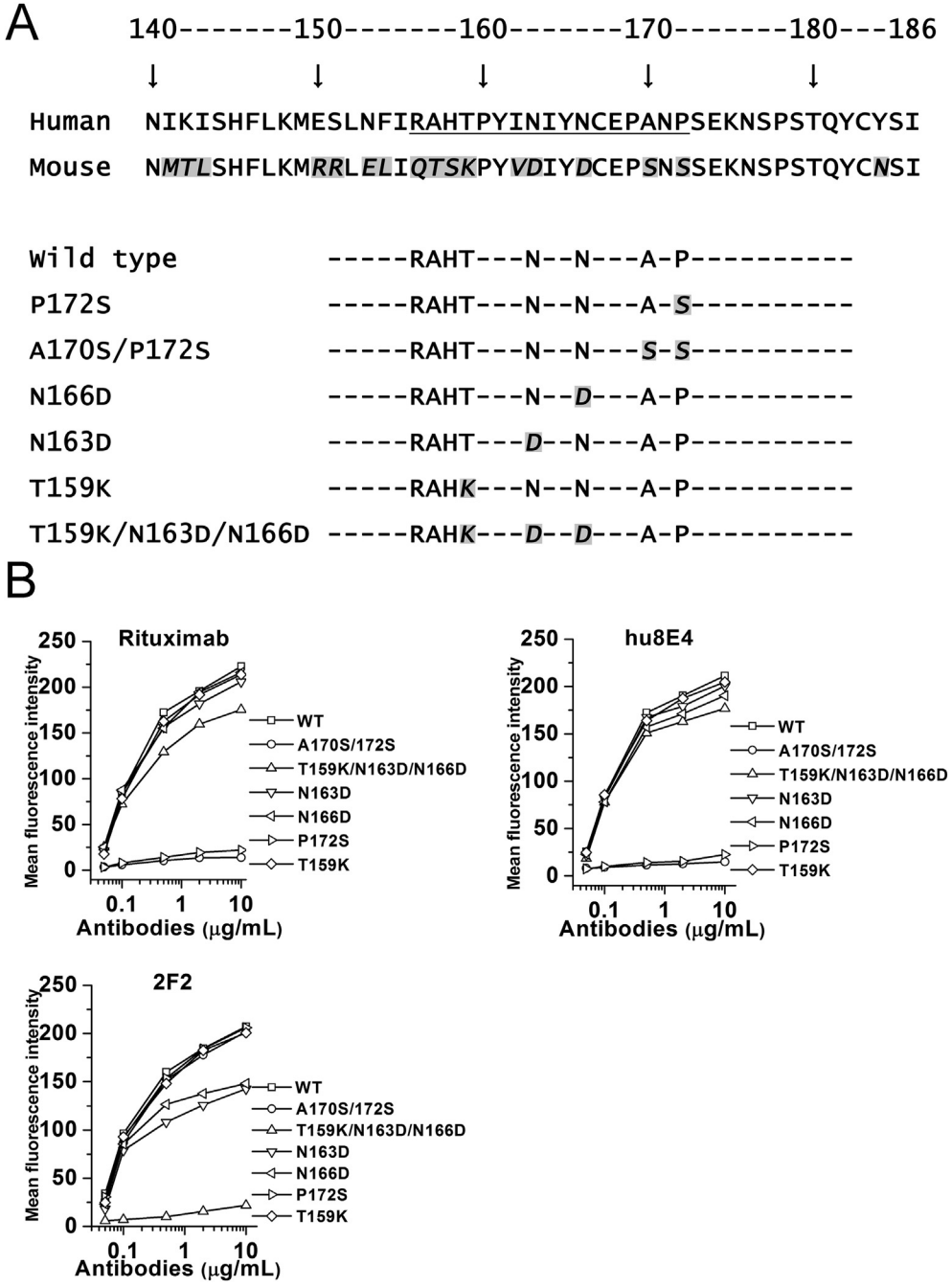


**Fig. 1.** The binding of Rituximab, hu8E4 and 2F2 to human CD20 and its mutants. (A) The predicted amino acid sequences for the extracellular regions of human and mouse CD20. Differences between the sequences are highlighted in gray. The human CD20 extracellular sequence (underline) was mutated to the murine sequence to generate human CD20 mutants P172S, A170S/P172S, N166D, N163D, T159K and T159K/N163D/N166D. Mutated residues in each human CD20 mutant are highlighted in gray. (B) Wild-type human CD20 and it mutants were expressed in CHO–K1 cells, and binding of anti-CD20 mAb to transfected cells was measured by flow cytometry.

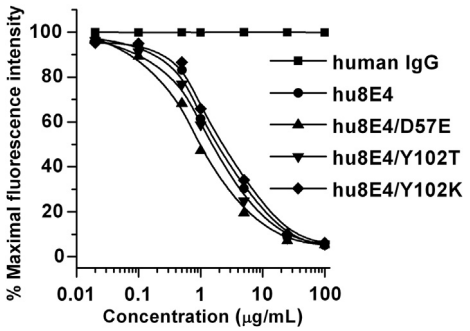


**Fig. 2.** Competitive binding assays of hu8E4 mutants. Raji cells were incubated with a subsaturating concentration of hu8E4-FITC and increasing concentrations of competing antibodies for 45 min at 4 °C. The cells were then washed and analyzed by FCM. Maximal fluorescence means the mean channel fluorescence obtained in the absence of competitor antibodies. All data were expressed as the mean of triplicate samples.

Rituximab was purchased from Roche Ltd. Eight-week-old female BALB/c SCID mice were housed in pathogen-free conditions and were treated in accordance with guideline of the Committee on Animals of Second Military Medical University.

### 2.2. Construction, expression, and purification

The anti-CD20 fully human antibody 2F2 and the anti-CD20 humanized antibody hu8E4 were expressed in CHO cells as described previously [13–15]. The recombinant antibodies were purified by affinity chromatography on Protein A-Sepharose (GE Healthcare). The point mutations were introduced into hu8E4 using overlapping PCR. Hu8E4 mutants were expressed and purified using the identical procedures described above.

### 2.3. Mutagenesis

The wild-type human CD20 cDNA was cloned into the pcDNA3.1(+) expressing vector (Invitrogen). Mutations in the extracellular regions of human CD20 were introduced using the QuickChange Multi Site-Directed Mutagenesis kit (SBS Genetech) according to manufacturer's instructions. The constructs were transiently transfected in CHO–K1 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. After , the transfected cells were used for flow cytometric binding experiments. Briefly, cells at $1 \times 10^6$ cells/mL were incubated with different concentrations of rituximab, 2F2 or hu8E4 for 1 h at 4 °C. The cells were washed and incubated with FITC-goat anti-human IgG (H + L) (Zymed) for 1 h at 4 °C. And then the cells were washed and analyzed by FCM.

### 2.4. Competitive binding assays

Raji cells at $1 \times 10^6$ cells/mL were incubated with a subsaturating concentration of the FITC-conjugated hu8E4 (hu8E4-FITC) and increasing concentrations of competing antibodies for 45 min at 4 °C. The cells were then washed and analyzed by FCM. The $IC_{50}$ values of competitors were calculated using a four-variable algorithm.

**Table 1**
Relative binding affinity of hu8E4 and its mutants.

| mAbs | $IC_{50}$ | SD |
|---|---|---|
| hu8E4 | 2.13 | 0.28 |
| $hu8E4_{D57E}$ | 1.45 | 0.21 |
| $hu8E4_{Y102T}$ | 1.67 | 0.35 |
| $hu8E4_{Y102K}$ | 2.48 | 0.42 |

### 2.5. Cytotoxicity assays

CDC and ADCC assays were performed as described previously [13]. Briefly, Raji or Daudi cells were incubated with mAbs for 1 h in phenol red-free PMRI-1640 culture medium in a 5% $CO_2$ 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 peripheral blood mononuclear cells (PBMCs) as effector cells (effector to target, 40:1 for ADCC assay). After an additional incubation for 4 h at 37 °C, the cell lysis was determined by measuring the amount of lactatedehydrogenase (LDH) released into the culture supernatant. Maximum LDH release was determined by lysis in 0.2% Triton X-100. Percentage of specific lysis was calculated according to the following formula: % lysis = [experimental release − spontaneous release]/[maximum release − spontaneous release] × 100.

### 2.6. Apoptosis assay

Raji or Daudi cells were incubated with different concentrations of CD20 mAbs at 37 °C for 16 h. After washing, cells were treated with annexin V-FITC (BD Biosciences), washed again, and analyzed by FCM.

### 2.7. Immunotherapy

Group of ten 8-week-old female SCID mice were injected via the tail vein with $5 \times 10^6$ Raji or Daudi cells on Day 0, followed 5 days later by the i.v. injection of 100 μg of CD20 mAbs. The mice were observed daily and euthanized at the onset of hind leg paralysis.

### 2.8. Statistical analysis

Statistical analysis was performed by Student's unpaired *t* test to identify significant difference unless otherwise indicated. Differences were considered significant at a *P* value of less than 0.05.

## 3. Results

### 3.1. Hu8E4 recognizes the A170/P172 motif

Previous epitope mapping studies of the extracellular loop of CD20 have indicated that A170 and particularly P172 are critical for recognition by rituximab and other tested anti-CD20 mouse mAbs, but not by the anti-CD20 human mAbs 2F2, 2C6 and 7D8 [12,16,17]. To examine whether A170 and P172 are also required for binding by hu8E4, we used site-directed mutagenesis to generate a set of human CD20 mutants, which were expressed in CHO–K1 cells. Then, we investigated the binding of rituximab, hu8E4 and 2F2 to these CD20 mutants. As shown in Fig. 1A and B, mutating the A170 and the P172 to serine (the mouse equivalent for these two amino acids) completely abolished the binding of rituximab and hu8E4. In fact, even a single mutation of P172 to serine was sufficient to totally abrogate the binding of these anti-CD20 mAbs. However, the A170S/P172S mutation had no influence on the binding of the anti-CD20 human antibody 2F2 (Fig 1A and B). In contrast, changing the N163 or N166 to the equivalent mouse residue markedly reduced the binding of 2F2 but had no effect on the binding of hu8E4 and rituximab (Fig. 1A and B). Next, we generated a CD20 triple mutant by mutating T159, N163 and N166 to the equivalent mouse residue. Our data showed that the binding of this triple mutant to rituximab

or hu8E4 was slightly weaker than that of wild-type CD20, but it totally lost the binding activity to 2F2 (Fig. 1A and B). Together, these data suggested that hu8E4 recognized epitope similar to that of rituximab.

### 3.2. Characterization of hu8E4 mutants

In the previous studies, we found that each of three single mutations (D57E, Y102K and Y102T) at the heavy chain variable region of rituximab improved the affinity of rituximab [10]. Here these three single mutations were introduced to the heavy chain variable region of hu8E4 to obtain three hu8E4 mutants, hu8E4$_{D57E}$, hu8E4$_{Y102K}$, and hu8E4$_{Y102T}$. The relative affinity difference between antibody and its mutant can be assessed by comparing the $IC_{50}$ values of them in the competitive binding assays. The results of the competitive binding assays indicated that all of the three hu8E4 mutants, hu8E4$_{D57E}$, hu8E4$_{Y102T}$ and hu8E4$_{Y102K}$, could compete with hu8E4 for binding to Raji cells (Fig. 2). As shown in Table 1, the affinity (mean $IC_{50} \pm SD$) of hu8E4 was lower than that of hu8E4$_{D57E}$ and hu8E4$_{Y102T}$ but higher than that of hu8E4$_{Y102K}$. Out of these hu8E4 mutants, hu8E4$_{D57E}$ exhibited the highest affinity.

### 3.3. CDC activity of hu8E4 mutants

The capacity hu8E4 mutants of to mediate CDC was assessed in two CD20-positive human lymphoma cell lines, Raji and Daudi. The results summarized in Fig. 3A showed that hu8E4$_{D57E}$, hu8E4$_{Y102T}$ and hu8E4$_{Y102K}$, exhibited approximately the same level of CDC activity as hu8E4. Moreover, hu8E4 and its mutants were significantly more potent in CDC than rituximab ($P < 0.05$ for rituximab

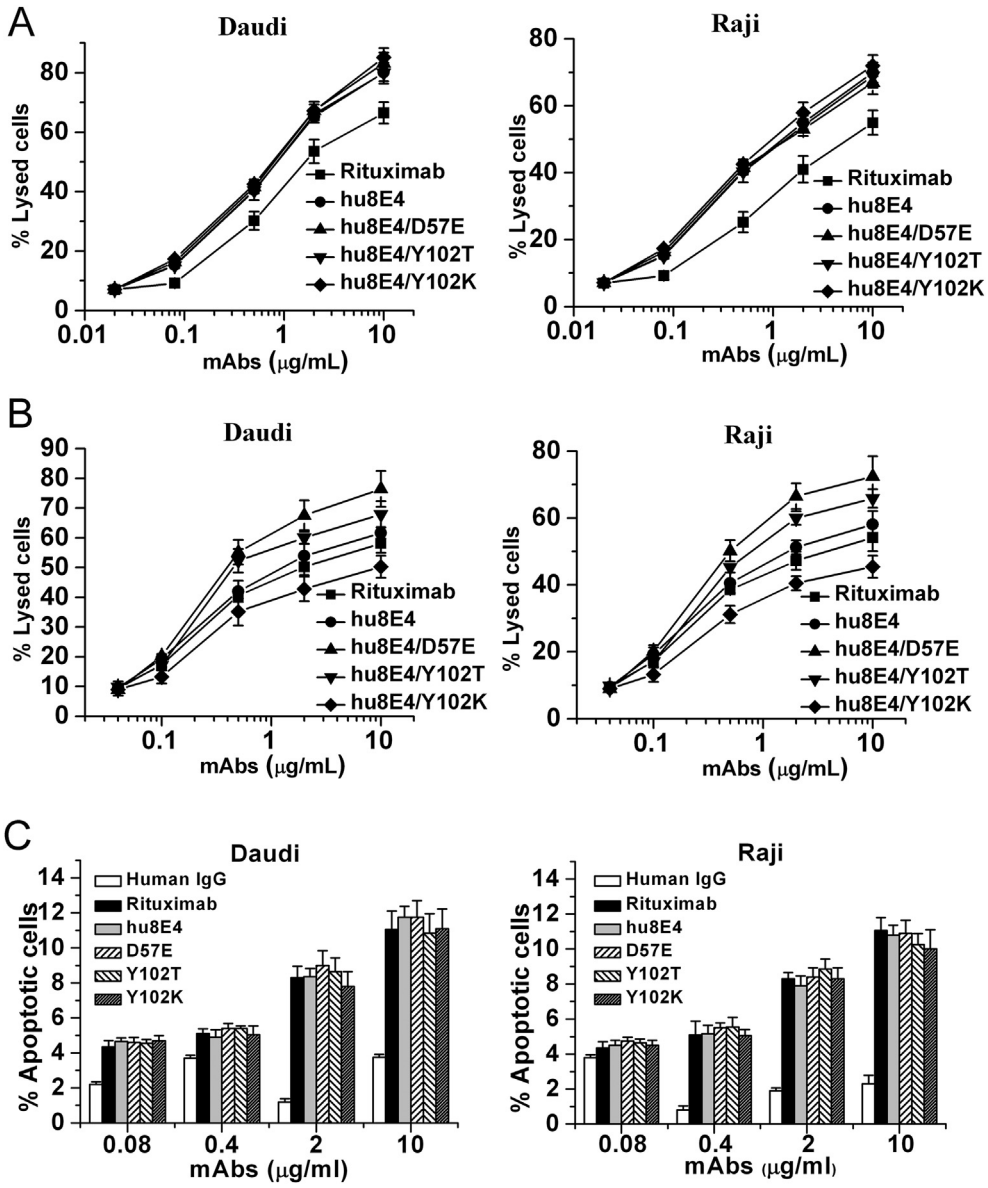


**Fig. 3.** CDC, ADCC and apoptosis induced by anti-CD20 mAbs. (A) Daudi and Raji cells were incubated with increasing concentrations of Rituximab, hu8E4, hu8E4$_{D57E}$, hu8E4$_{Y102K}$ and hu8E4$_{Y102T}$ in the presence of human complement at 37 °C for 4 h. CDC activity of these antibodies was measured by the CytoTox 96 non-Radioactive Cytotoxicity Assay kit. Data are expressed as means ± SD (n = 3). (B) Daudi and Raji cells were incubated with increasing concentrations of Rituximab, hu8E4, hu8E4$_{D57E}$, hu8E4$_{Y102K}$ and hu8E4$_{Y102T}$ in the presence of human PBMCs at 37 °C for 4 h. ADCC activity of these antibodies was measured by the CytoTox 96 non-Radioactive Cytotoxicity Assay kit. Data are expressed as means ± SD (n = 3). (C) Daudi and Raji cells were incubated with different concentrations of Rituximab, hu8E4, hu8E4$_{D57E}$, hu8E4$_{Y102K}$ and hu8E4$_{Y102T}$ at 37 °C. Apoptosis was assessed 16 h later by Annexin V staining. Apoptotic cells were measured as percentage of total cells assayed. Columns, mean (n = 3); Error bars, SD.

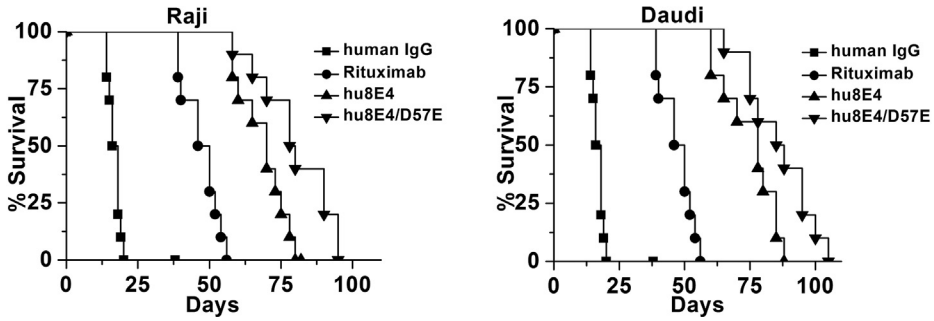


**Fig. 4.** The survival of tumor-bearing SCID mice treated with anti-CD20 mAbs. Groups of 10 SCID mice were injected i.v. with $5 \times 10^6$ Raji or Daudi cells. Five days after tumor cell inoculation, the mice were treated with 100 μg of anti-CD20 antibodies or the human IgG control. Mice were monitored daily and sacrificed at the onset of hind leg paralysis.

compared with each of hu8E4, hu8E4$_{D57E}$, hu8E4$_{Y102T}$ and hu8E4$_{Y102K}$ at concentrations of 0.08, 0.4, 2 and 10 μg/mL (Fig. 3A).

### 3.4. ADCC activity of hu8E4 mutants

The ability of hu8E4 mutants to lyse CD20-positive human lymphoma cells in the presence of human PBMCs was investigated and compared with hu8E4. The results shown in Fig. 3B indicated that all of these anti-CD20 antibodies effectively mediated ADCC against Daudi or Raji cells in a dose-dependent manner. Hu8E4 and rituximab exhibited similar ADCC activity, which is consistent with the results reported previously [13]. Moreover, our data showed a clear affinity-dependent susceptibility to ADCC. Hu8E4$_{D57E}$, which had the highest affinity, exhibited the most potent ADCC Activity (Fig. 3B).

### 3.5. Apoptosis-inducing activity of hu8E4 mutants

Induction of apoptosis was evaluated by FITC-Annexin V assays in Daudi and Raji cells. As shown in Fig. 3C, all of the three hu8E4 mutants, hu8E4$_{D57E}$, hu8E4$_{Y102T}$ and hu8E4$_{Y102K}$, were able to trigger low levels of apoptosis of both of these two CD20-positive lymphoma cells, and their apoptotic activity appeared to be similar to that of hu8E4 and rituximab.

### 3.6. Therapeutic efficacy of hu8E4 mutants

The therapeutic efficacy of hu8E4 mutants was evaluated in in SCID mice bearing systemic Daudi or Raji tumors. (SCID/Daudi and SCID/Raji). The survival curves were plotted according to Kaplan–Meier method and compared using the log-rank test. The results summarized in Fig. 4 indicated that all of the anti-CD20 antibodies, rituximab, hu8E4 and hu8E4$_{D57E}$ significantly improved the survival of SCID mice bearing disseminated Raji tumor cells ($P < 0.001$ for each compared with the human IgG control). Hu8E4 and hu8E4$_{D57E}$ were shown to be markedly more effective than rituximab in prolonging the survival of SCID/Raji mice ($P < 0.001$ for either Hu8E4 or hu8E4$_{D57E}$ compared with rituximab). Moreover, a pronounced difference in survival was observed between Hu8E4 and hu8E4$_{D57E}$ treatment groups ($P = 0.0161$), and hu8E4$_{D57E}$ showed more potent antitumor activity (Fig. 4). Similar results were obtained using Daudi tumor-bearing SCID mice (Fig. 4).

## 4. Discussion

The A170/P172 motif within the large extracellular loop of CD20 is critical for recognition by rituximab [12]. Our present studies have demonstrated that A170 and P172 are also required for binding by hu8E4, suggesting that the epitopes recognized by rituximab and hu8E4 are very similar. In our previous studies, three single mutations (D57E, Y102T and Y102K at the heavy chain variable region of rituximab) were proved to be able to improve the affinity of rituximab [11]. In this study, these three single mutations were transferred to hu8E4, and we found that D57E and Y102T but not Y102K successfully enhanced the binding of hu8E4 to CD20. This raises a question why Y102K fails to improve the affinity of hu8E4. Hu8E4 is as effective as rituximab in mediating ADCC and inducing apoptosis in B-lymphoma cells, but it exhibits much more potent CDC than rituximab [13]. These data suggest that although both rituximab and hu8E4 recognize the A170/P172 motif, epitope heterogeneity still exists between these two mAbs. This may explain why the single mutation Y102K that can improve the affinity of rituximab reduces the binding of hu8E4.

Consistent with the results reported previously [13], hu8E4, which is significantly more potent in CDC than rituximab, exhibits markedly higher in vivo antitumor activity compared with rituximab. These data supports an important role for complement in CD20 mAb immunotherapy. Furthermore, hu8E4$_{D57E}$ appears to be significantly more effective in prolonging the survival of tumor-bearing SCID mice than hu8E4. Since hu8E4$_{D57E}$ displays a similar ability to mediate CDC and to induce apoptosis against B-lymphoma cells compared with hu8E4, it can be concluded that the enhanced in vivo antitumor effect of hu8E4$_{D57E}$ may be attributable to its markedly increased ADCC activity.

In conclusion, the data shown here suggest that the mutations that can improve the affinity of rituximab may be transferred to other anti-CD20 murine mAbs to enhance their binding to CD20. The anti-CD20 humanized antibody hu8E4$_{D57E}$, which exhibits more potent antitumor activity than that of hu8E4, may be a promising therapeutic agent for the treatment of B-cell lymphomas.

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## Conflict of interest

The authors declare that there are no conflicts of interest.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.158.

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