Supplementary Data 1: C3B Solution Competition Assay

ELISA microplate wells were coated using 5 μg/ml of protein sample as indicated in Results with or without 30 KU/ml CA125, and with or without 5 μg/ml C3B or C4B in 0.05 M carbonate buffer at pH 9.5. After overnight incubation at 4˚C, wells were washed three times with PBS at pH 7.2. Non-specific binding sites were blocked using PBS containing 5% BSA for 1h at room temperature. Coated protein samples were detected by using 2.5 μg/ml of biotinylated antibody as indicated in Results in PBS containing 0.5% BSA for 1 h at room temperature. After washing, 0.3 μg/ml of HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc.; cat. no. 016-030-084) was added to samples and incubated for 1 h at room temperature. After washing, TMB substrate (Thermo Fisher Scientific, Inc.; cat. no. 34029) was added and incubated for up to 30 min at room temperature and the colorimetric reaction was stopped using 0.1N H_2SO_4. Absorbance was read at 450 nm using Varioskan plate reader and using SkanIt™ version 4.1 (Thermo Fisher Scientific, Inc.).

Figure S1. (A) Twelve random RTX HC variant clones were sequenced to determine mutation frequency. Number of mutated bases in the RTX HC coding sequence ranged from 0 to 13, with an average number of mutations per RTX HC clone of 4.75, thus allowing an adequate degree of mutation diversity within the library. In addition, one clone with 4 and another with 13 mutated bases also had 1 and 2 deleted bases, respectively, leading to open reading frame shift and predicted truncation of the HC. These data suggest that approximately 90% of the library included full-length heavy chain variants. HC, heavy chain; RTX, rituximab. (B) Twelve random rituximab heavy chain variant clones were sequenced and the different possible base changes were ranked based on number of occurrences. The data show a high degree of base change diversity.

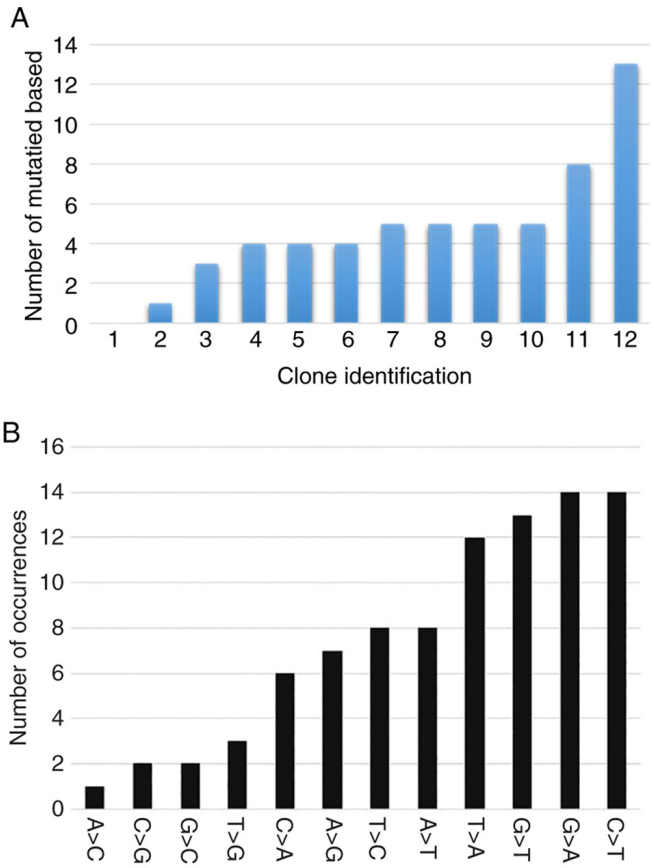

Figure S2. Comparison of 5 lead RTX variants from the library screening based on CA125 binding normalized to CD20 binding. RTX, rituxamib; WT, wild type.

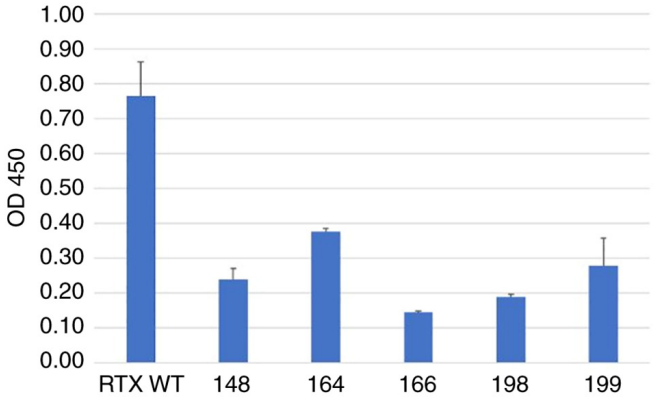

Figure S3. Comparison of 5 lead rituxamin variants from the library screening based on least reduced CDC activity in the presence of CA125. CA125, cancer antigen 125; CDC, complement-dependent cytotoxicity.

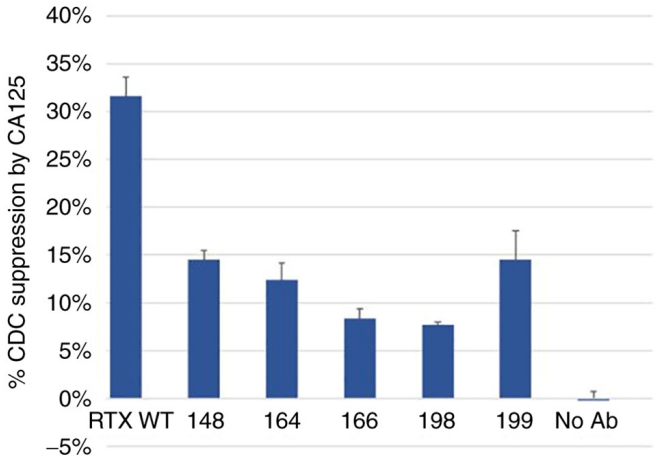

Figure S4. (A) Alignment of RTX and RTX N109D nucleotide and amino acid sequences immediately surrounding the N109D change. (B) Alignment of PTZ and RTX heavy chain of complementarity determining region 3 sequences. PTZ, pertuzumab; RTX, rituximab.

Figure S5. Potential immunogenicity mediated by T-cell epitopes using well-established algorithms showed that the RTX N109D has equivalent or reduced immunogenicity score associated to peptides within the complementarity determining region 3/framework 4 region compared to parent RTX. RTX, rituximab.

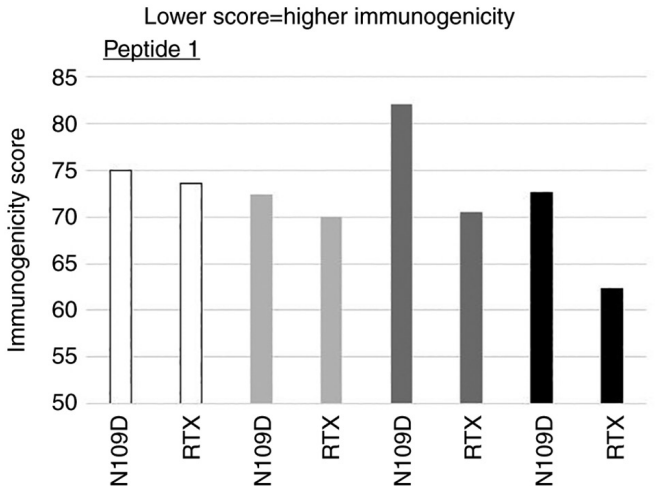

Table SI. RTX variants library screening summary.

Number of variants at start	Primary ELISA hits	Secondary ELISA hits	Top lead variants	Validated lead
204 (RTX-001 to RTX-204)	30	11	5 (Figs. S2 and S3)	RTX-166

RTX, rituximab.

Table SII. Identification of RTX HC-CDR3 sequence according to various definitions.

Definition	HC-CDR3	Residue ID	Number of residues
Chothia	--STYYGGDWYFNV	99-110	12
AbM	--STYYGGDWYFNV	99-110	12
Kabat	--STYYGGDWYFNV	99-110	12
Contact	ARSTYYGGDWYFN-	97-109	13
IMGT	ARSTYYGGDWYFNV	97-110	14

HC-CRD3, heavy chain of complementary determining region 3.