CD20 peptide synthesis for Pepscan library
A total of 4291 primarily CLIPS peptides were synthesized based on the published CD20 sequence (CD20_HUMAN, Prim. accession # P11836, 227 residues).

The peptide libraries were focused on the following extracellular loop regions:
Pos 142–187: KISHFLKMESLNFIARAHTPYINIYNCEPANPSEKNSPSTQYCYSIQ
Pos 72–80: IPAGIYAPI
Pos 167–183: CEPANPSEKNSPSTQYC

The CLIPS peptides were synthesized as described previously. Three different CLIPS topologies were used: T2 CLIPS couple to the side-chain of two cysteines to form a single loop topology. T3 CLIPS couple to the side-chain of three cysteines to form a double loop topology. T2T3 CLIPS combine T2 (labeled “C”) and T3 CLIPS (labeled “1”) to give triple looped topology.

The following peptide libraries were synthesized:
(A) All overlapping linear 15-mer peptides covering complete CD20 (Pos 1–297).
(B) same peptides as A) but in single looped topology. The side-chains of two cysteines were coupled to the T2 CLIPS.
(C) All 34-mers and an alanine-scan of each of these 34-mers covering the long extracellular loop of CD20 (Pos 142–187). The two cysteines were allowed to oxidize through their cysteines.
(D) All 7-mers covering Pos 142–187 as XXXXXXX in triple-looped peptides of either “C”(Pos 72–80)“1”(Pos 168–182)-C-XXXXXX or “C”(Pos 72–80)-“1”XXXXXXX-C(PoS 168–182) topology.
(E) A full positional scan of Pos 167–183, in which each residue was replaced by all possible other natural amino acids (excluding cysteine).
(F) All 34 overlapping 15-mers covering Pos 142–189 plus Pos 72–80 synthesized in a 35×35 matrix in double-looped topology C-X15-C-X15-C.
(G) All 40 overlapping 15-mers covering Pos 142–188 plus Pos 72–80 synthesized in a 40×40 matrix in triple-loop topology 1C-X9-1-X9-C-X9-1.

Site-directed mutagenesis
The following forward primers were used for site-directed mutagenesis:
C167S: 5′-ACATATACAACACTGACAACCAGCTAATCC-3′
E168A: 5′-CATATACAACCTGTGCACCAGCTAATCCCTCTG-3′
N171A: 5′-TGTGAACCAGCTGCACCCCTCTGAGAAAAC-3′
N171V: 5′-TGTGAACCAGCTTACCTCTGAGAAA-3′
N171S: 5′-TGTGAACCAGCTCCACCCTCTGAGAAAAC-3′
N171H: 5′-TGTGAACCAGCTCACCCCTCTGAGAAAAC-3′
N171P: 5′-TGTGAACCAGCTTACCCCTCTGAGAAAAC-3′
N176A: 5′-CCCTCTGAGAAAGCTTCCCCATCTACC-3′
S177A: 5′-TCTGAGAAAAACGCTCCGTGACCAGCTACGT-3′
Testing mRNA and protein levels of CD20 variants

qRT PCR reactions were performed in triplicates in a 96 well plate. 1 µl cDNA synthesis product derived from 1 µg of total RNA was used per reaction. With the LightCycler® 480 Probes Mastermix (Roche) amplification was performed over 40 cycles of 10s 95°C / 30s 60°C / 60s 72°C. Amplification of CD20 and the house-keeping gene glucuronidase beta were simultaneously monitored from each well using probe 50 and GUSB probe.

Transiently transfected HEK293 cells were lysed on day 2 post transfection with RIPA lysis buffer (Thermo Scientific). Precleared whole cell lysates of each sample (10µg/lane) were separated on NuPAGE 12% polyacrylamid gels (Invitrogen). After transfer to nitrocellulose with the iBlot system (Invitrogen), the CD20 variants were immunodetected using either a rabbit antibody directed against a carboxyterminal peptide of CD20 (Cat.# 1632-1, Epitomics) or a rabbit anti-HA tag antibody from Serotec (Cat.# AHP1075G).

Crystallography

A Fab fragment of GA101 was prepared by papain digestion for 3 hours. The Fc portion was removed using a HiTrap MabSelect Xtra column and the Fab fragment purified by gel filtration on a Sephadex 75 column. Purity and homogeneity were confirmed by analytical SEC and SDS PAGE analysis.

GA101 Fab was crystallized using hanging drop vapor diffusion at 20°C. 1µl of protein (24.9 mg/ml in 20 mM His-HCl pH 6.0 / 140 mM NaCl) was mixed with 1µl reservoir solution (0.1 M Hepes pH 7.5 / 24% (w/v) PEG4000 / 0.15 M ammonium sulfate). Crystals were cryoprotected by adding reservoir solution supplemented with 20% (v/v) D-(–)-2,3-butanediol. Diffraction data were processed with XDS2 to 2.5 Å resolution. Using the structure of a humanized anti–IFN-Gamma Fab fragment (PDB ID 1T04) as search model, PHASER3 was able to locate 2 molecules in the asymmetric unit. Manual model building was performed using COOT 4 and the structure was refined with PHENIX5 including bulk solvent corrections, individual B-factor refinement, translation-libration-screw– and positional refinement.

After introducing a disulfide bond between Cys167 and Cys183 the following peptide derived from the larger extracellular loop of CD20 was used:

\[163\text{NIYNCEPANPSTQYCSIQ}^{187}\]. For complex formation, the epitope peptide was dissolved at a 10:1 molar ratio in GA101 Fab solution (8 mg/ml in 20 mM His-HCl pH 6.0 / 140 mM NaCl). Crystals grew at 20°C in hanging drops after mixing 1µl of the GA101 Fab:CD20 peptide complex with 1µl reservoir solution (16% (w/v) PEG4000 / 4% (v/v) isopropanol / 0.1 M sodium acetate). For cryoprotection crystals were equilibrated overnight against reservoir solution containing 35% (w/v) PEG4000. Diffraction data were processed to a resolution of 1.6 Å with XDS and the GA101 Fab structure was used for molecular replacement with PHASER. Refinement was carried out according to the GA101 Fab refinement. Data collection and refinement statistics are summarized in Table 1.

Elbow angles for GA101 were calculated using RBOW.6 Buried surfaces were calculated with AreaIMol.7 Figures were prepared with PYMOL.8 Coordinates have been deposited at the Protein Data Bank (accession numbers 3PP3 for the apo structure and 3PP4 for the peptide bound structure).
Isothermic calorimetry
GA101 (17 μM ≈ 34 μM binding sites) and rituximab (60.5 μM ≈ 121 μM binding sites) were extensively dialyzed against PBS buffer (10 mM Na₂HPO₄, 1 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, sterile filtered and degassed). The peptide was dissolved into dialysis buffer and adjusted to 500 μM (or 2000 μM for measurements using rituximab). After equilibrating the cell of a VP-ITC device (Microcal LLC, Northhampton, US) to the corresponding temperature, 10 μl sample injections were done 30 times spaced by 3 min. Data was evaluated using Origin-based Microcal evaluation software with manual baseline selection. Concentration data of the antibody is always depicted as concentration of binding sites.

Protein tomography
Ramos RA1 cells either untreated or preincubated with GA101 or rituximab were fixed with 4% PFA by adding an equal volume of double strength fixative to the culture medium. After 10 min at room temperature, the cells were harvested and resuspended in fresh single strength fixative. After incubation at room temperature for 2 hours the cell pellet was rinsed in 0.1 M phosphate buffer and shipped to the Cell Microscopy Center at UMC, Utrecht, for further preparation for cryosectioning. After blocking of nonspecific antibody binding sites, the sections were immunolabelled with rabbit anti-human IgG and protein A-gold followed by contrasting, and embedding of specimen sections in polyvinyl alcohol. For protein tomography analysis, the immunolabeling was first evaluated under conventional EM conditions, i.e. analyzed at 80 kV accelerating voltage. For tomography data collection, Formvar-coated parallel bar grids (Cu, 150 lines, Agar Scientific, England) carrying 10 nm colloidal gold particles sandwiched in a double layer of carbon were used. These larger gold particles were subsequently used for the geometrical alignment of the tilt series (alignment gold). Tilt series of micrographs were recorded at 2° tilt intervals in the range ±60° and at 24,000× total magnification, giving a final pixel size of 5.73 Å. Defocus was set at −1 μm. In total 25 tilt series were recorded of the GA101-treated cells and 15 tilt series of the rituximab-treated cells. A post-experimental high-dose, large under-focus, micrograph was recorded on the same area after each tilt series. The average alignment error was 6.4 Å for the GA101 tilt series and 6.7 Å for the rituximab tilt series. Alignment gold, marker gold, and cell membranes were readily visible in the post-experimental highdose micrographs. The micrograph recorded at 0° tilt angle was used to locate and select marker gold for 3-D reconstruction. The tomograms were reconstructed by a conventional filtered back projection technique to obtain initial densities. Refinement using the patented Sidec COMET algorithm was run for 30 iterative cycles to improve the signal-to-noise ratio. At the location of selected marker gold, tomograms of sizes between 170×170×170 pixels and 300×300×300 pixels were computed from the micrographs. Only one tilt-series of each antibody sample was excluded due to bad quality.

Labeling of GA101 and rituximab with ALEXA 488 and ALEXA 546
The two monoclonal antibodies were coupled to the respective fluorescent dye using the Invitrogen antibody labeling kits (A20181 and A20183). The average dye/protein ratio was 2.5 for GA101_ALEXA546 and 6.2 for rituximab_ALEXA488, respectively.
REFERENCES

**Figure S1. Control experiments for the FACS binding studies with different CD20 variants**

(A) qRT-PCR results demonstrating comparable mRNA levels of all transiently transfected CD20 variants. (B) Western blots of whole cell lysates with anti-CD20 (I) and anti-HA (II) antibodies showed comparable protein levels for wildtype and mutant CD20 variants with two exceptions: (i) the C167S variant (labeled “SEP”) was barely detectable in all experiments, probably due to misfolding and proteolytic degradation. (ii) for unknown reasons the S177A variant (labeled “NAP”) was in several independent experiments always present in higher levels than wildtype CD20.

**Figure S2. Additional protein tomography results**

(A–F) illustrate the different steps in the protein tomography™ analysis of cell sections: (A) is a two-dimensional electron micrograph recorded with high electron dose and underfocus settings to obtain a high contrast image. In those regions, where the plasma membrane is oriented perpendicular to the section cut, it can be seen as two parallel dark lines. The cytoplasms of cells are observed as darker regions compared to the extracellular space. The larger black dots (some marked by black arrows) are 10 nm colloidal gold particles embedded in the grid. These marker gold dots are used to align the different micrographs from a tilt series. The red circle marks a 5 nm protein A-conjugated gold particle (immunogold marker), which identifies the membrane proximal location of a bound anti-CD20 antibody. Based on a tilt series of micrographs, a 3D tomogram is reconstructed around the immunogold marker labelled in (A) (for details see methods section above). (B) shows this 3D tomogram superimposed on the high contrast electron microscopic image from (A). (C) is a zoomed in and tilted view of the same tomogram as shown in (B). A white arrow marks the electron density that represents the immunogold marker labelled by a red circle in (A). (D) represents a rotated cut-out of the tomogram showing the immunogold (elongated spherical density volume on the right) and a connected macromolecular complex. The structures bound to the immunogold appear as an assembly of partly merged, repeating, volume entities with similar shapes. (E) is a color annotated zoom-in version of the same tomogram. The part of the complex assigned as the primary antibody, in this case GA101, recognized by the secondary anti-human Ab conjugated to the gold particle, is colored blue. The protein complex binding to GA101, in this case a CD20 tetramer, is shown in red. (F) represents a cut-out of the CD20 tetramer showing only one of the volume entities. These entities often consisted of two connected parts whose combined size correlates well to that estimated for CD20. The larger parts, to which the antibodies bound, were interpreted as the extracellular and transmembrane portion, and the smaller parts were assigned as the intracellular regions of CD20. (G) shows a tomogram that is representative of a rituximab bound, large protein network. Similar large protein densities were not seen in any of the tomograms associated with GA101. (H) is a color annotated version of the same tomogram. The parts of the tomogram representing the immunogold are shown in grey and the protein network, including rituximab, is shown in red.