Supplemental Material

CD22 induced B-cell tolerance prevents inhibitory antibodies to FVIII

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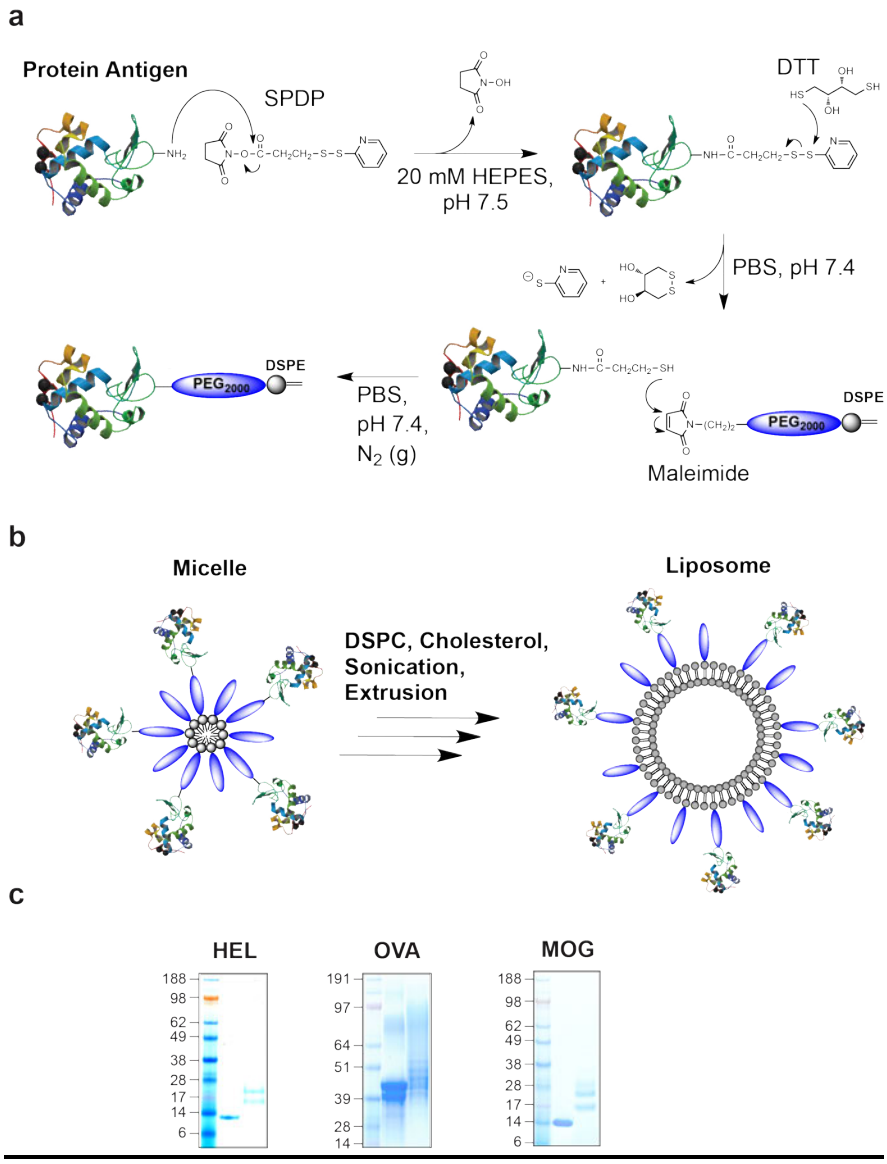

Figure S1
Biochemical conjugation strategy for coupling of proteins to pegylated lipids. **(A)** Proteins were first reacted with the heterobifuncational crosslinker SDSP. Following deprotection to produce a free thiol, the protein is reacted with maleimide-PEG$_{2000}$-DSPE. **(B)** The protein conjugated to pegylated lipid exists as micelles in solution and used to hydrate the appropriate mixture of dried lipids. Rigorous sonication followed by extrusion produces uniform 100 nm stealth liposomes displaying the antigen. **(C)** SDS-PAGE analysis of proteins linked to pegylated lipid. Each gel is loaded as follows; lane 1: protein later, lane 2: unmodified protein, lane 3: modified protein. All three proteins had between 1-3 lipids attached per protein.

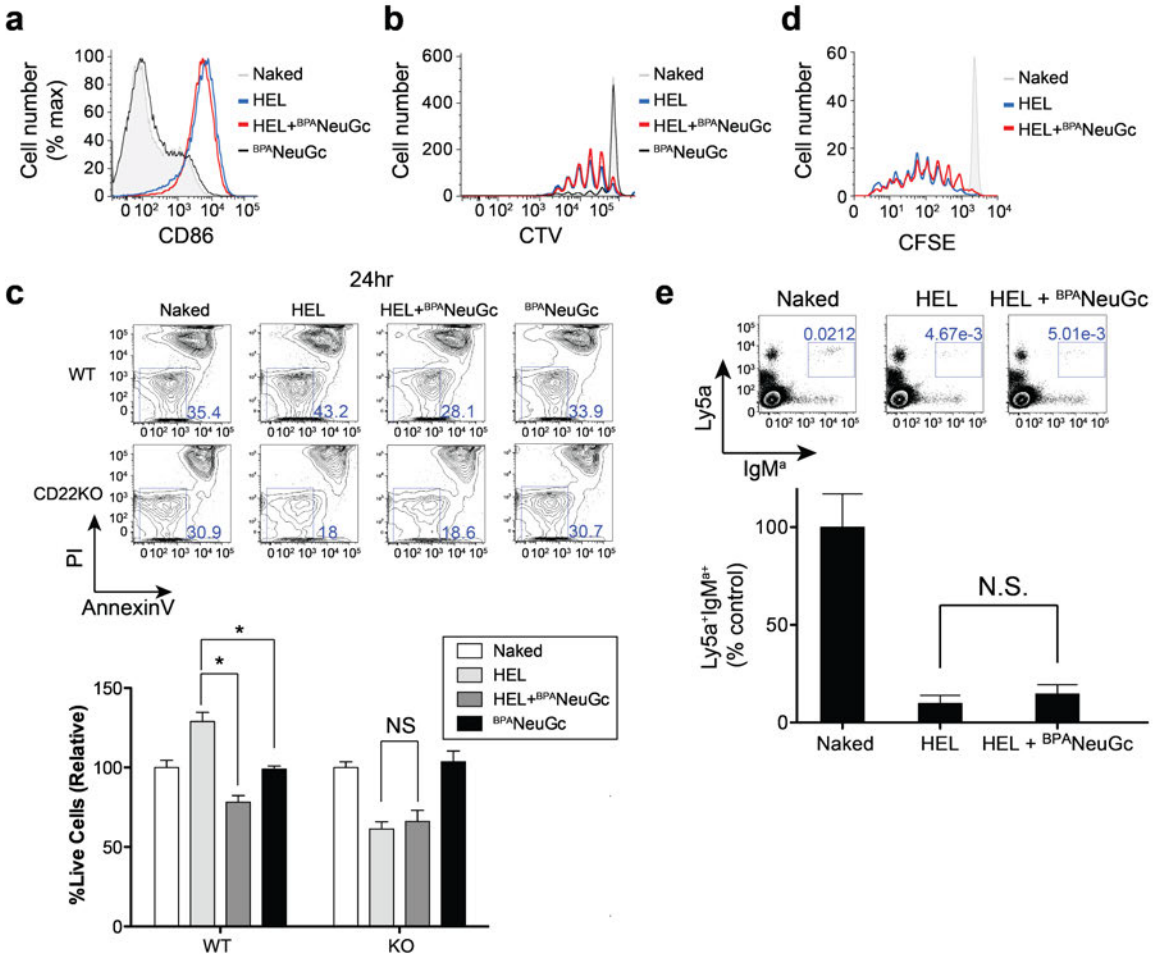

Figure S2

CD22-dependence of results using STALs displaying HEL and IgM^{HEL} B-cells. **(A)** CD22KO IgM^{HEL} B-cells were stimulated with the indicated liposomes *in vitro* for 24hr and activation was assessed by CD86 expression by flow cytometry. **(B)** CTV-labeled CD22KO IgM^{HEL} B-cells were stimulated with the indicated conditions *in vitro* for 3 days and cell proliferation was determined by CTV dilution by flow cytometry. **(C)** WT or CD22KO IgM^{HEL} B-cells were incubated with the indicated liposomes for 24 hr. Cells were stained with PE-AnnexinV and PI to assess cell viability. For quantitation, the number of live cells (PI$^-$AnnexinV$^-$) was standardized to 100% for cells treated with naked liposomes. Quantitation represents mean +/- s.e.m. **(D)** CFSE-labeled CD22KO IgM^{HEL} B-cells (5x10^6) were adoptively-transferred into host mice and then stimulated with the indicated liposomes the following day. Four days later, CFSE dilution of the IgM^{a+}Ly5^{a+} cells was determined by flow cytometry. **(E)** Ablation of IgM^{HEL} B-cells *in vivo* by treatment with STALs is CD22-dependent. Ly5a$^+$IgM^{HEL} B-cells (2x10^6) on a CD22KO background were adoptively-transferred into recipient mice. One day later, mice were immunized with the indicated conditions and the spleens were analyzed 12 days later. Quantitation represents mean +/- s.e.m. (n=5). There is no statistical difference (N.S.; $P > 0.05$) in the number of Ly5a$^+$IgM^{a+} cells recovered from mice immunized with immunogenic versus tolerogenic liposomes. All data is representative of three independent replicates.

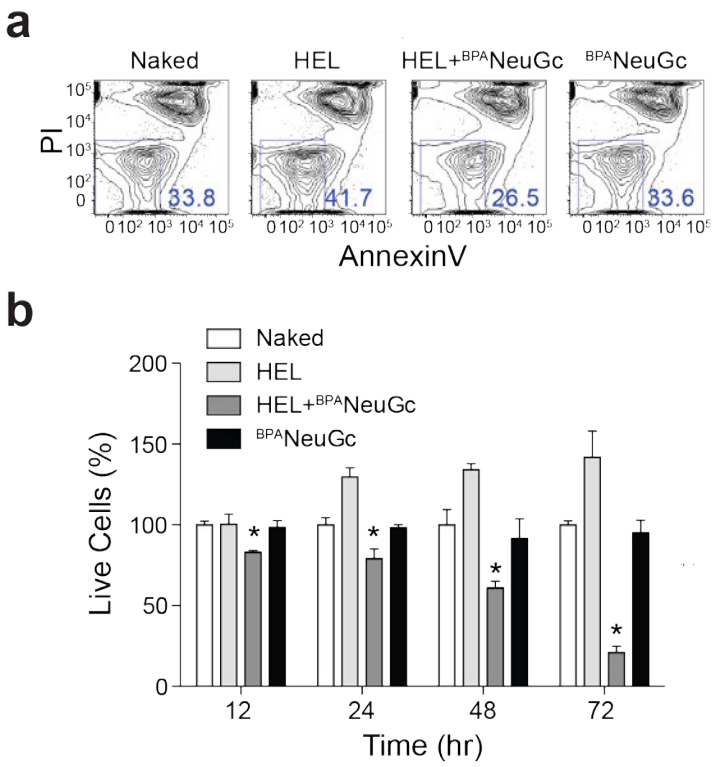

Figure S3
IgM^{HEL} B-cells incubated with STALs show a time-dependent decrease in cell viability even in the presence of anti-CD40. **(A)** IgM^{HEL} B-cells were cultured with the indicated liposomes for 24 hours. Cells were stained with PE-AnnexinV and PI and analyzed by flow cytometry. Data is representative of three replicates. **(B)** The same experiment was conducted at various time points. For comparison of the different treatments, the $\%PI^{-}AnnexinV^{-}$ (live) cells are normalized to the control at treated with naked liposomes (set to 100% at each time point), and plotted as the mean +/- s.e.m. (n=3). Quantitation of the number of cells through the $PI^{-}AnnexinV^{-}$ gate. Data represents mean +/- s.e.m. (n=3). The asterisks represent a statistical difference (P < 0.05) from the other groups.

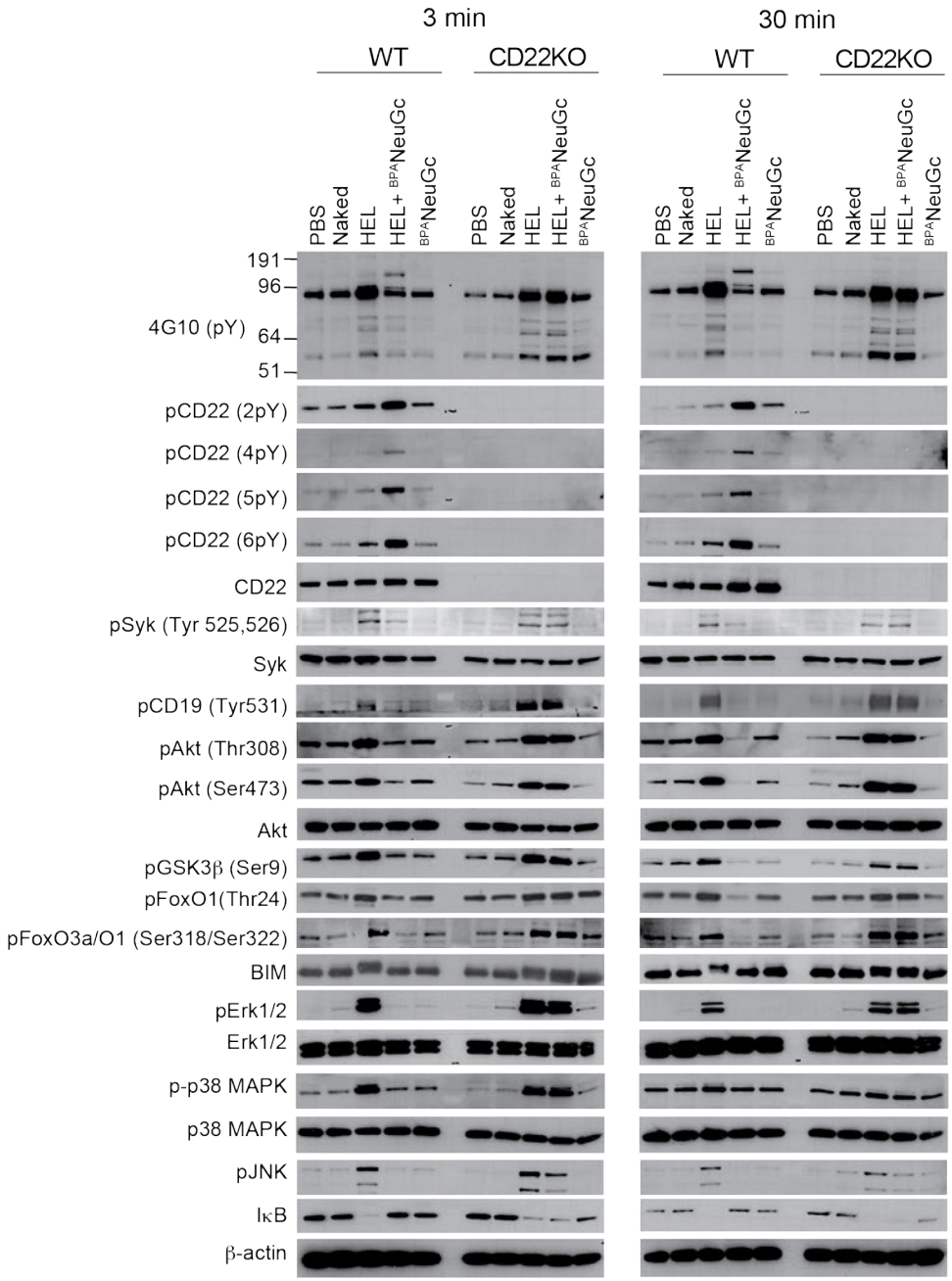

Figure S4 Analysis of BCR signaling components following stimulation with STALs. Western blot analysis of BCR signaling components in WT and CD22KO IgM^{HEL} B-cells 3 and 30 minutes after stimulation of cells with the indicated liposomes or PBS as a control. STALs inhibit phosphorylation of signaling components of all major BCR signaling pathways and induce hypophosphorylation of Akt and FOXO in WT B-cells. CD22-deficient IgM^{HEL} B-cells demonstrate the CD22-dependence of the effects. Results are representative of three independent experiments.

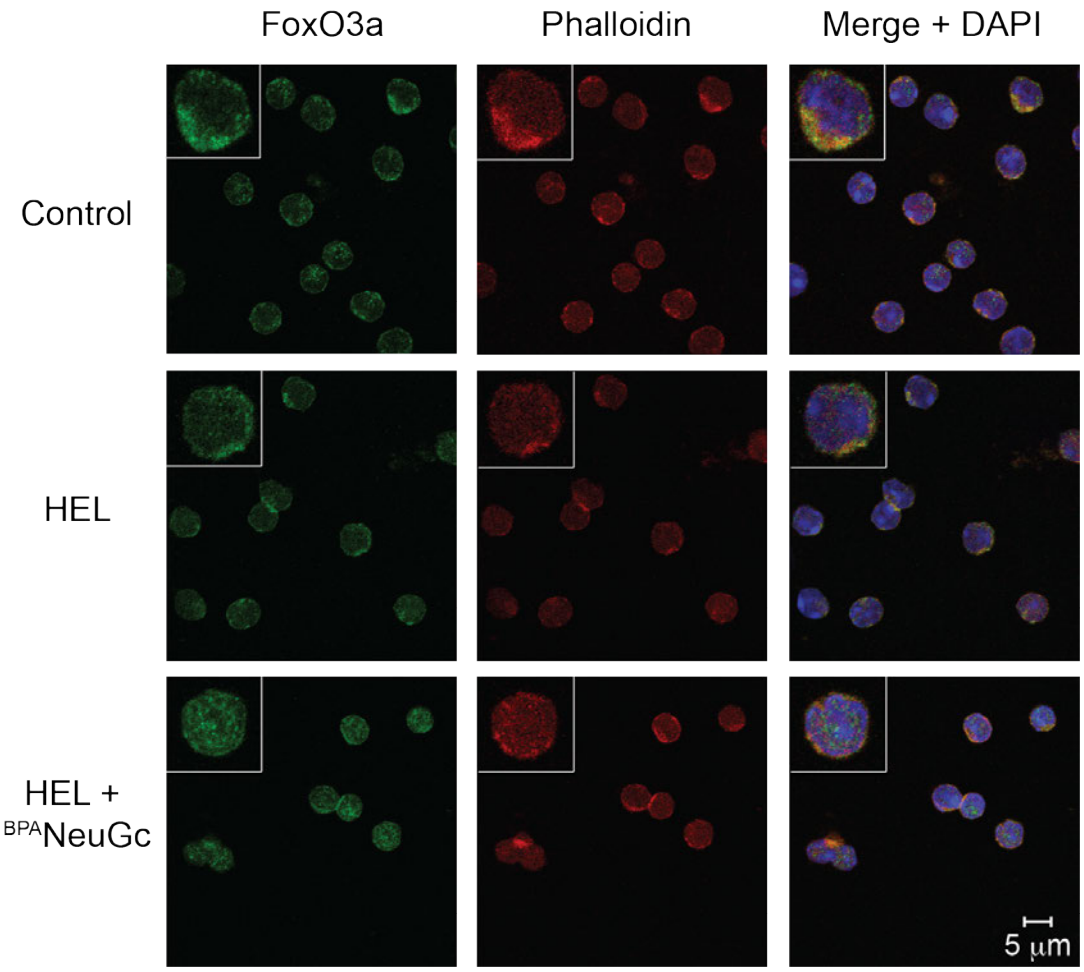

Figure S5.
STALs induce nuclear localization of the FoxO3a transcription factor. Confocal microscopy of WT IgM^{HEL} B-cells stimulated with the indicated liposomes for 2 hr. Cells were stained with anti-FoxO3a, phalloidin, and DAPI. Inserts are a representative cell at three-times the magnification.

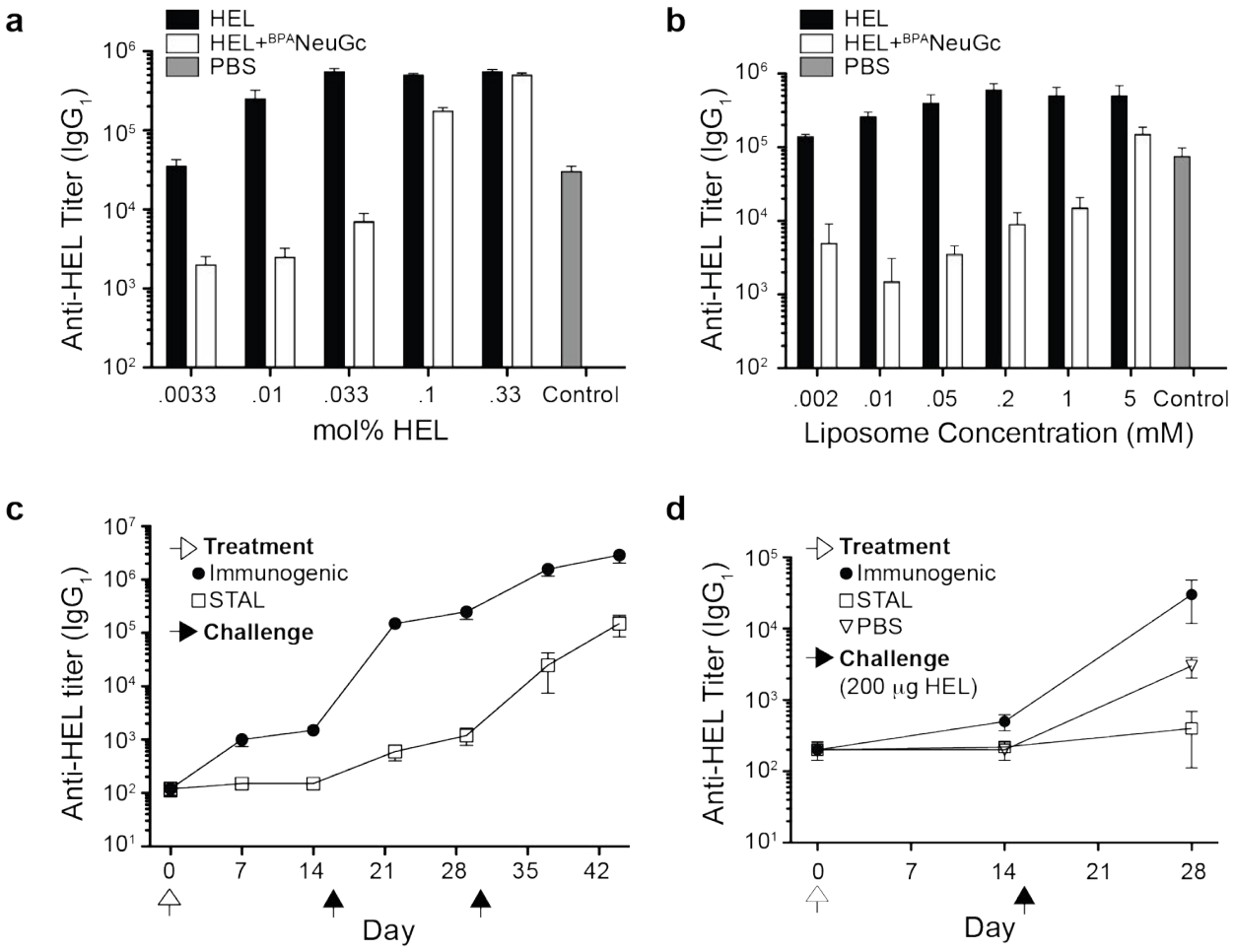

Figure S6

Optimization of the STAL formulation enables tolerization of Balb/c mice to HEL. **(A)** Optimization of the mol% of HEL on liposomes given to Balb/c mice. Mice were immunized on day 0 with 200 μL of 1 mM of liposomes containing the indicated mol % of HEL with (open bars) or without (black closed bars) ^{BPA}NeuGc, challenged on day 15 with immunogenic liposomes, and titers determined two weeks later on day 29. **(B)** Optimization of the amount of liposomes given to Balb/c mice. Mice were immunized on day 0 with the indicated concentration of 200 μL of liposomes containing 0.01 mol% HEL with (open bars) or without (black closed bars) ^{BPA}NeuGc, challenged on day 15 with immunogenic liposomes, and titers determined two weeks later on day 29. **(C,D)** Using optimized conditions (0.01 mol%, 10 μM) STALs induce tolerance of HEL in Balb/c mice to a liposomal (**C**) or soluble (**D**) challenge. Mice were immunized on day 0 with the indicated conditions, challenged on day 15, and titers determined two weeks later on day 29. All data represents mean +/- s.e.m. (n=4).

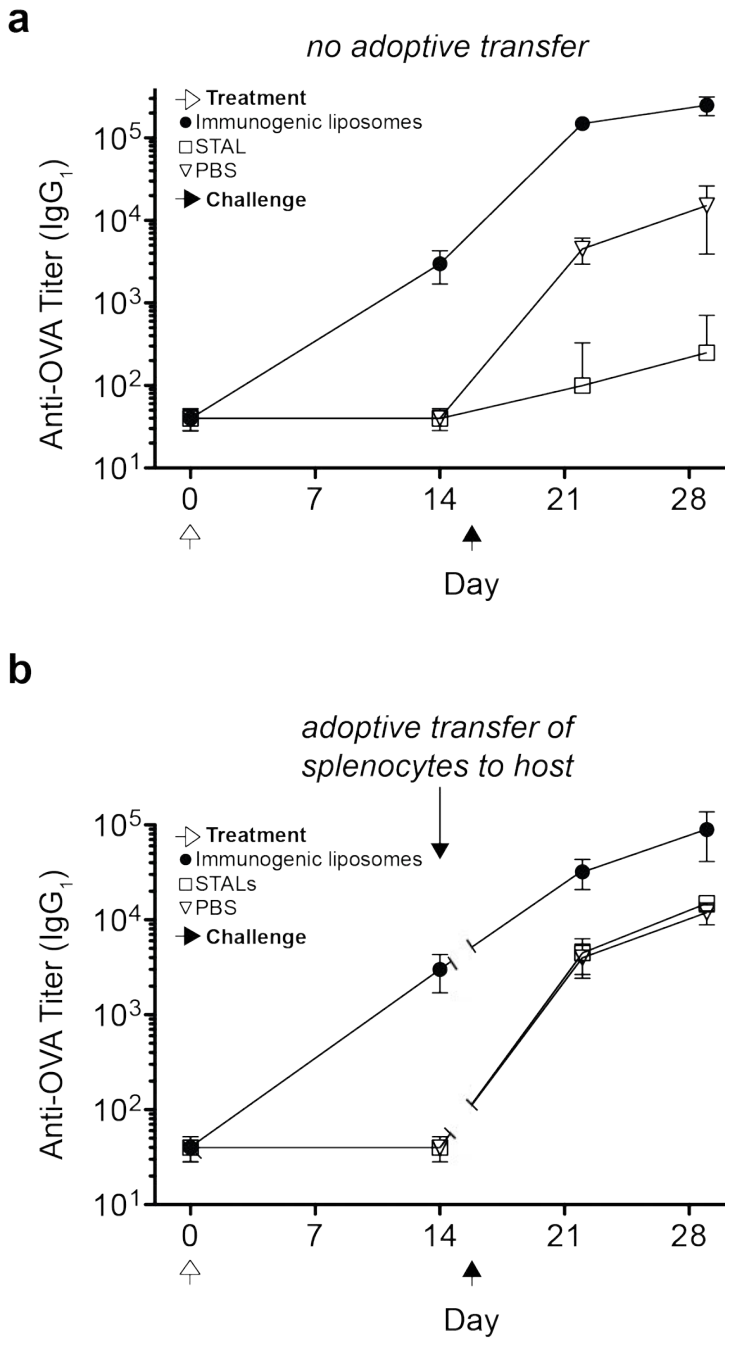

Figure S7
Transfer of splenocytes from a tolerized mouse does not suppress an antibody response in host mice. **(A)** on Day 0, three groups of C57BL/6J mice were treated with either liposomes displaying OVA alone (filled circles), liposomes displaying OVA and ^{BPA}NeuGc (empty squares), or vehicle (PBS; empty triangles). All three groups were challenged with immunogenic liposomes on day 15 (as denoted by solid arrow). **(B)** Same experiment as above except on day 14, the spleens of mice from all three groups were harvested and $20x10^6$ splenocytes injected into host mice. The following day (day 15), all three groups were challenged with immunogenic liposomes (as denoted by solid arrow). Data represents mean +/- SEM (n=5).

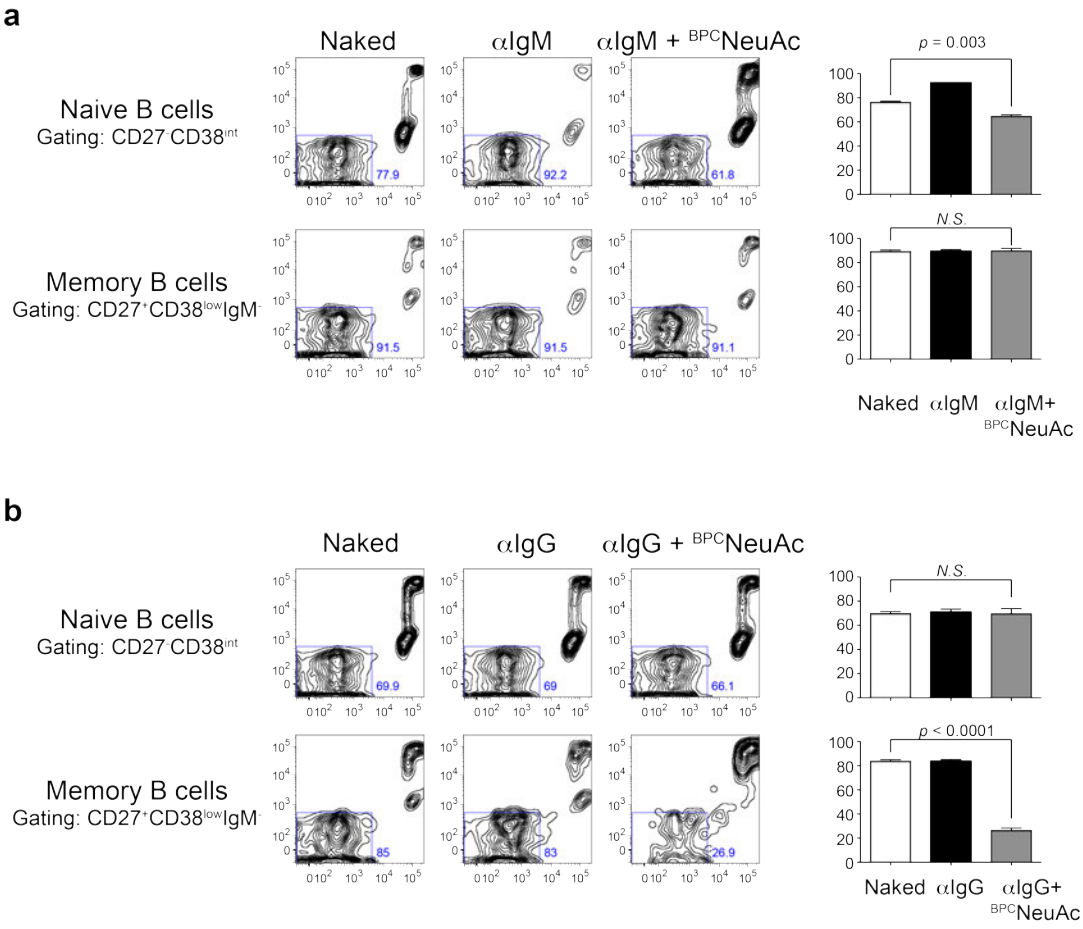

Figure S8
Apoptosis of human naïve and memory B-cells requires simultaneously display of CD22 ligands and cognate antigen. **(A,B)** Purified human primary B cells were incubated with the indicated liposomes. PI and AnnexinV staining was analyzed on the two major B cell populations: Naïve and isotype-switched (IgM^-) memory B cells, using the indicated gates. **(B)** Data represents mean +/- SEM (n=5). *N.S.* represents no statistical significance. The top panel of **(A)** and bottom panel of **(B)** are Figure 6E in the main text.