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Supplementary Materials for

The Scaffolding Protein Synapse-Associated Protein 97 Is Required for Enhanced Signaling Through Isotype-Switched IgG Memory B Cell Receptors

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Other Supplementary Material for this manuscript includes the following:
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Movies S1 to S4 (.avi format)

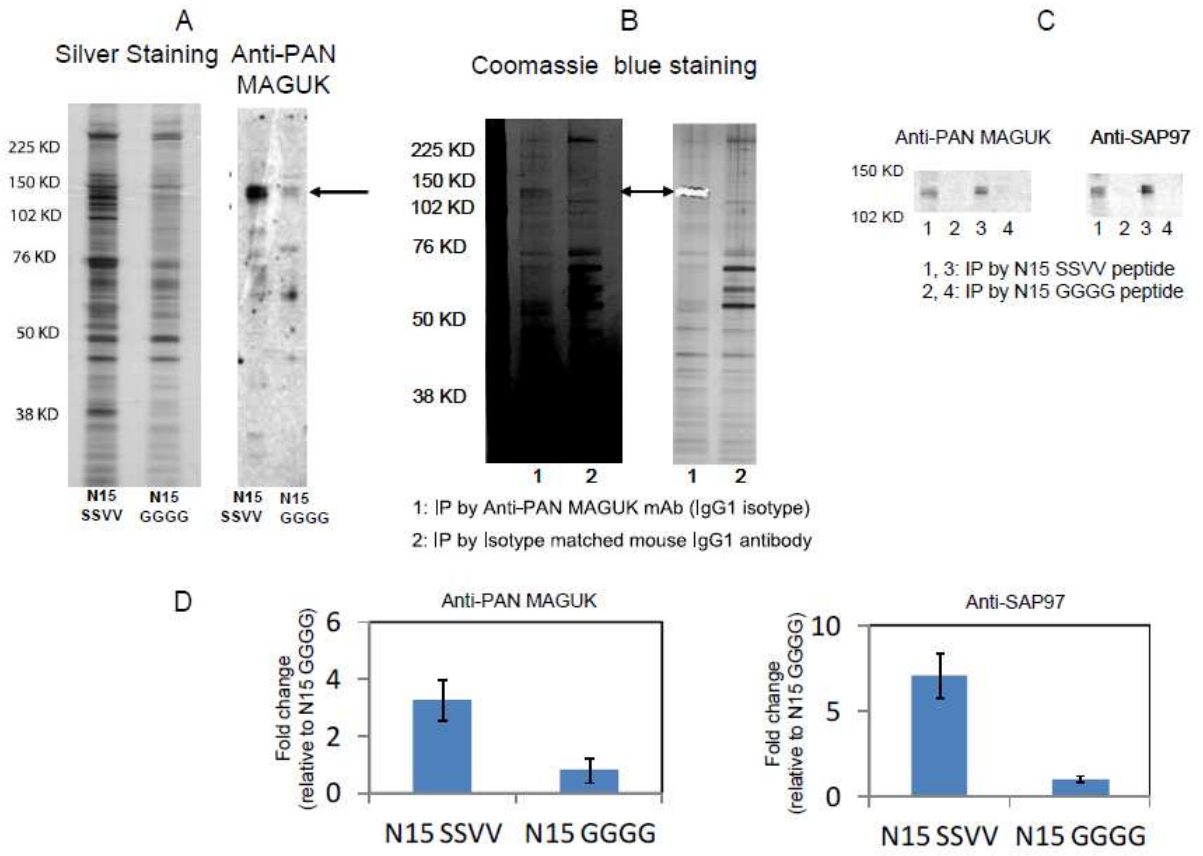

Fig. S1. The cytoplasmic tail of mIgG binds to SAP97. (**A**) Silver staining (left) and Western blotting analysis with anti-PAN MAGUK mAb (right) of SDS-PAGE gels of samples immunoprecipitated from J558L cell lysates with a biotin-conjugated membrane-proximal 15 amino acid peptide that contained the SSVV motif (N15 SSVV) bound to streptavidin beads or with a biotinylated control peptide in which SSVV was changed to GGGG (N15 GGGG) bound to streptavidin beads. The black arrow indicates the 130-kD protein recognized by the anti-PAN MAGUK antibody. (**B**) Coomassie blue staining of samples immunoprecipitate from J558L cell lysates with beads conjugated to an anti-PAN MAGUK mAb or to an isotype matched control antibody. On the left is the original SDS-PAGE gel stained with coomassie blue, on the right is the gel after removal of the 130-kD protein band indicated by the black arrow that was subjected to LC-MS/MS analysis by ProtTech. (**C**) Western blotting analysis of Samples immunoprecipitated from A20 II1.6 cell lysates with either N15 SSVV peptide or N15 GGGG control peptide were analyzed by Western blotting with either anti-PAN MAGUK mAb (left, Clone K28/86) or anti-SAP97 mAb (right, Clone K64/15). Immunoprecipitates (IPs) 1 and 2 and IPs 3 and 4 were from independent lysate preparations loaded onto the same SDS-PAGE gel. (**D**) Statistical analysis of the mean fold-change ± SD in the intensity of the N15 SSVV bands relative to those of the N15-GGGG control samples from four independent experiments.

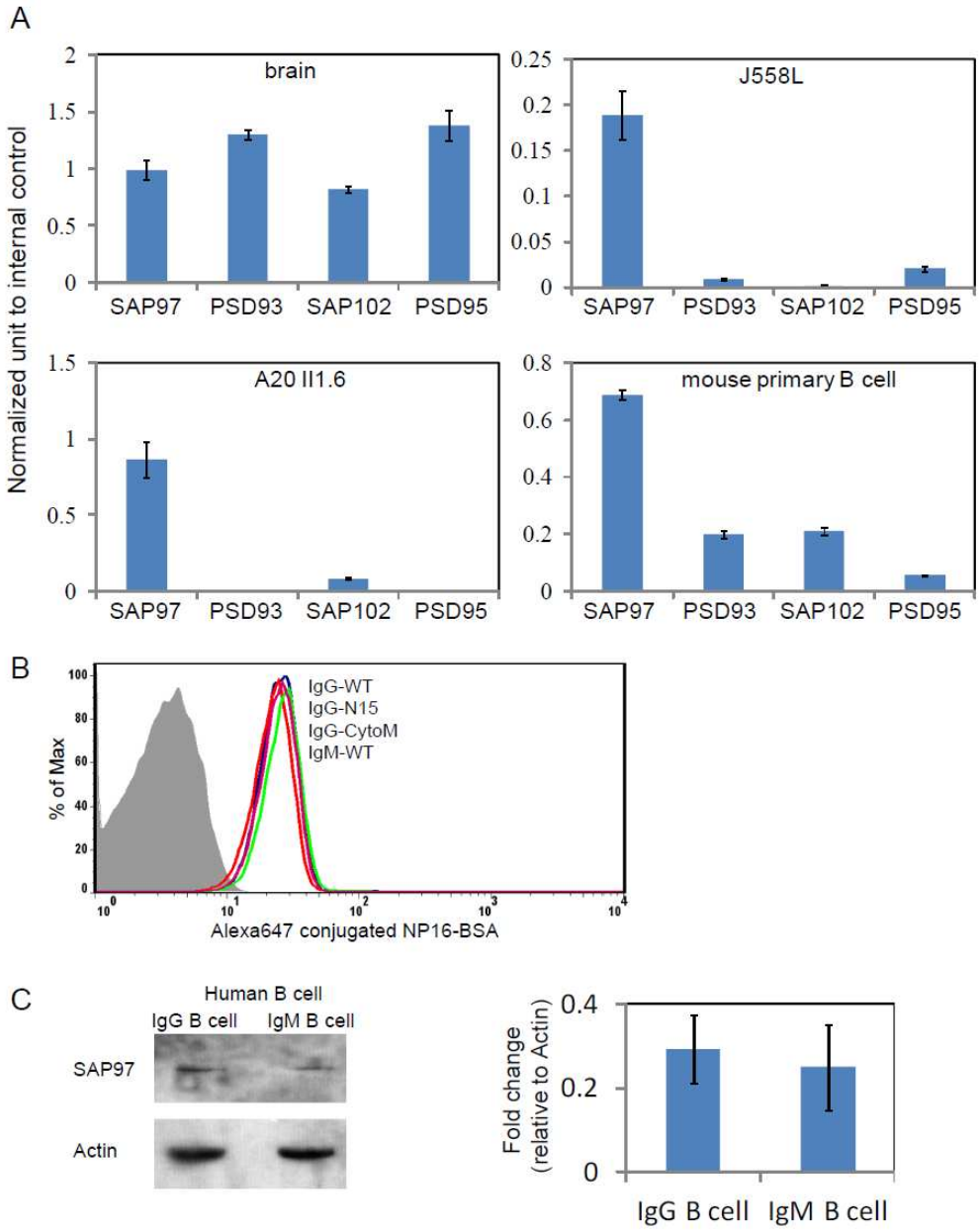

Fig. S2. SAP97 is the most abundant SAP family protein in B cells. (**A**) Quantitative RT-PCR analysis of the abundances of mRNAs for the four SAP family members in brain homogenate, J558L cells, A20 II1.6 cells, and freshly purified primary mouse splenic B cells. Real-time RT-PCR primers specific for *Dlg1* (SAP97), *Dlg2* (PSD93), *Dlg3* (SAP102), *Dlg4* (PSD-95), and internal control were purchased from Qiagen and were used according to the manufacturer's instructions. Arbitrary units for the abundance of the mRNA for each SAP family member normalized to that of the internal control are given. Brain samples have large amounts of all four SAP family members and were therefore used as controls of primer quality and the experimental system. (**B**) Cell-surface abundances of nitrophenol (NP)-specific BCRs (either of the IgG or IgM type) in J558L cells expressing IgG-WT, IgG-N15, IgG-CytoM, or IgM-WT were measured by flow cytometric analysis Alexa Fluor 647–conjugated NP16-BSA staining. (**C**) Human IgM- and IgG-expressing B cells both express SAP97. Primary human IgM- and IgG-expressing B cells sorted from the peripheral blood of healthy donors were lysed and subjected to SDS-PAGE and Western blotting analysis for human SAP97. The data represent the mean ± SD from two independent experiments.

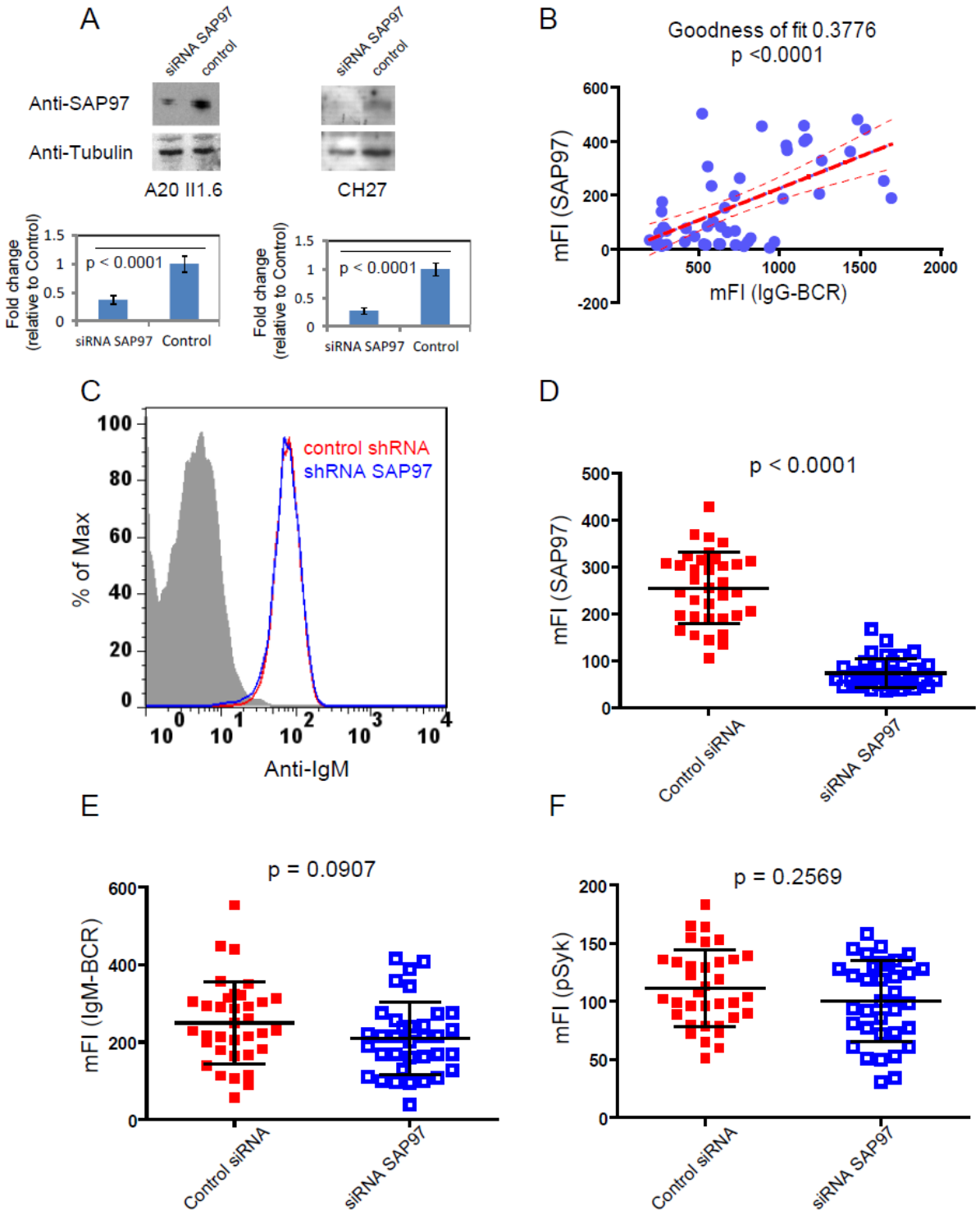

Fig. S3. Knockdown of SAP97 has mild effects on the antigen-induced accumulation of IgM BCRs in the immunological synapse. (**A**) Western blotting analysis of the presence of SAP97 in A20 II1.6 cells (left) and CH27 cells (right) tranfected with a thermal smart pool siRNA specific for SAP97 (left) or with pSuper-shRNA-SAP97 (right) as well as of their corresponding control constructs. Bar graphs show the fold-change in SAP97 abundance relative to that of control cells

as well as the statistical analysis. Data are means ± SD from three independent experiments. (**B**) The correlation of the MFI of IgG-BCRs with that of SAP97 within the immunological synapse of IgG^+ B cells. (**C**) Cell-surface abundance of IgM-BCRs in CH27 control cells or CH27 stable sub-lines in which SAP97 was knocked down were measured by flow cytometric analysis of Alexa Fluor 647–conjugated anti-mouse IgM staining. The MFIs of (**D**) SAP97, (**E**) BCR, and (**F**) pSyk within the IS of CH27 control cells or CH27 stable sub-lines in which SAP97 was knocked down. Each dot represents one cell analyzed in at least three independent experiments, and the bars represent means ± SD. Two-tailed *t* tests were performed for the statistical comparisons.

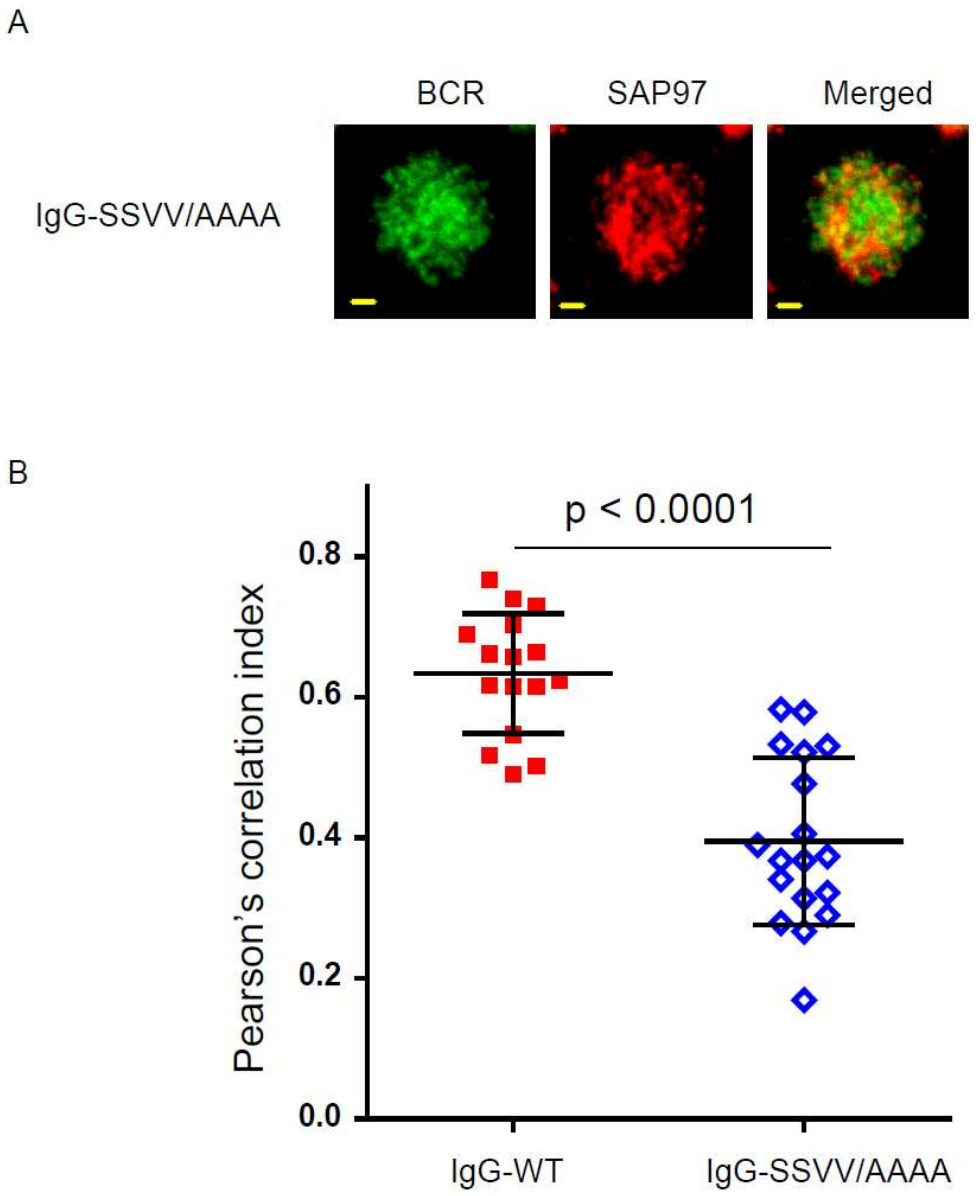

Fig. S4. The extent of colocalization of IgG-SSVV/AAAA mutant BCRs with SAP97 upon antigen engagement is substantially reduced compared to that of IgG-WT BCRs. (**A**) Two-color TIRF images of the BCR and SAP97 within the immunological synapses of J558L cells expressing IgG-SSVV/AAAA BCRs. The cells were placed on planar lipid bilayers containing NIP-H12 for 10 min, fixed, permeabilized, and then incubated with fluorescently tagged antibodies specific for the BCR or for SAP97. Scale bar: 1.5 µm. (**B**) The Pearson's correlation index was applied to quantify the colocalization of BCR and SAP97 within the immunological synapase for cells expressing IgG-WT or IgG-SSVV/AAAA BCRs. Each dot represents one cell analyzed in two independent experiments, and bars represent means ± SD. Two-tailed *t* tests were performed for the statistical comparisons.

Movie descriptions

Movie S1. Representative 3D images showing the distribution of the BCR on J558L cells expressing reconstructed NP-specific IgG-BCRs that were placed on planar lipid bilayers containing no antigen. The 3D images are shown in three different views: side view, top view, and 45° angle bird view.

Movie S2. Representative 3D images showing the distribution of SAP97 on J558L cells expressing reconstructed NP-specific IgG-BCRs that were placed on planar lipid bilayers containing no antigen. The 3D images are shown in three different views: side view, top view, and 45° angle bird view.

Movie S3. Representative 3D images showing the accumulation and distribution of the BCR in J558L cells expressing reconstructed NP-specific IgG-BCRs that were placed on planar lipid bilayers containing NIP-H12 antigen. The 3D images are shown in three different views: side view, top view, and 45° angle bird view.

Movie S4. Representative 3D images showing the accumulation and distribution of SAP97 in J558L cells expressing reconstructed NP-specific IgG-BCRs that were placed on planar lipid bilayers containing NIP-H12 antigen. The 3D images are shown in three different views: side view, top view, and 45° angle bird view.