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Figures and figure supplements

B cell activation involves nanoscale receptor reorganizations and inside-out signaling by Syk

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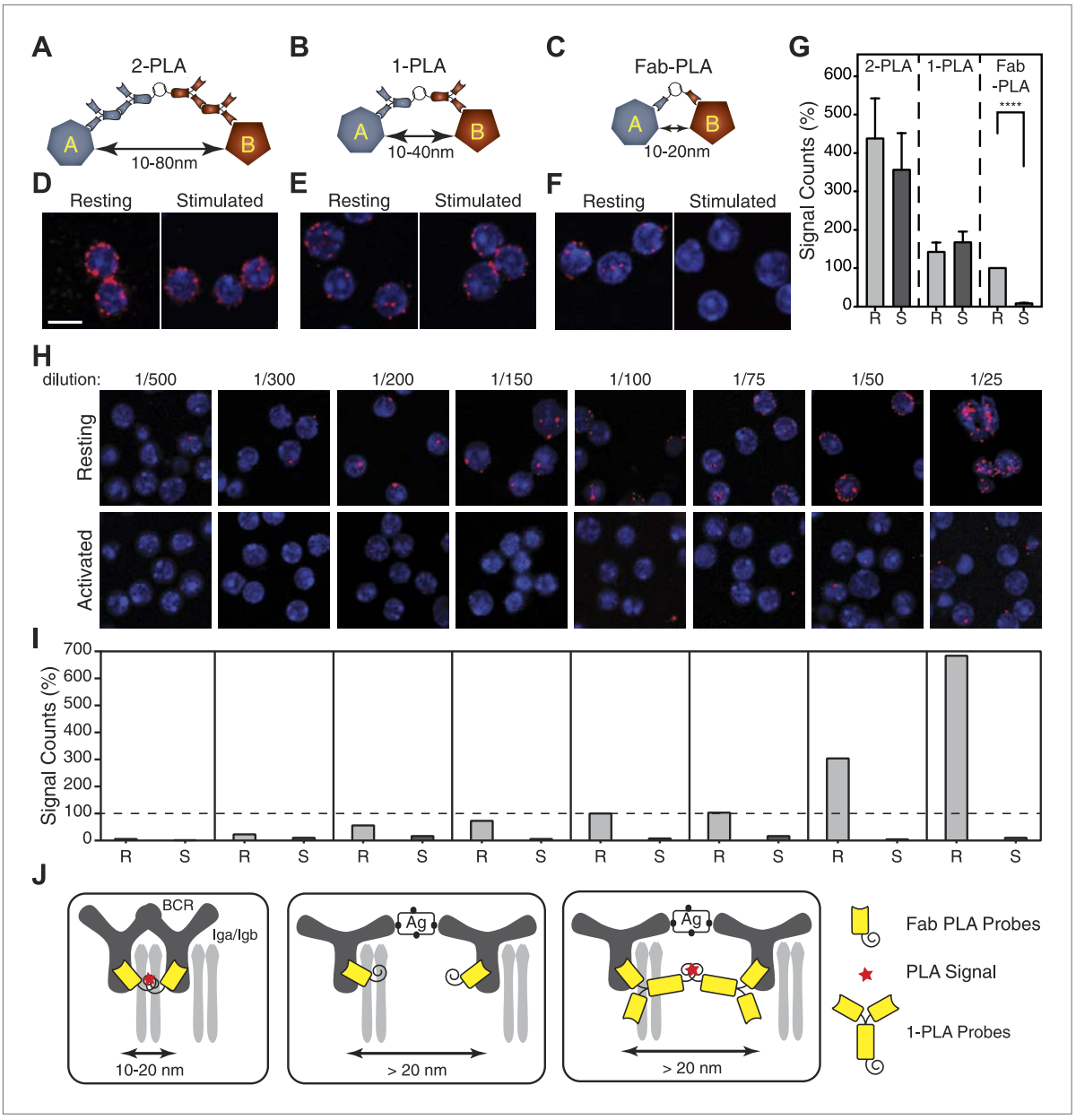

Figure 1. Nanoscale analysis of the IgM:IgM BCR proximity on the surface of TKO-MD B cells by PLA of different settings. (**A**–**C**) Scheme of the three PLA settings. Plus and minus oligos for annealing and rolling circle amplification were coupled to either (**A**) secondary antibodies (2-PLA), (**B**) primary antibodies (1-PLA) or (**C**) Fab fragments (Fab-PLA). The theoretical detection ranges are noted under the double-heads-arrows for each setting. (**D**–**F**) Representative confocal microscopic images of IgM:IgM PLA of resting (left) and antigen (NIP-BSA) activated (right) TKO-MD cells by (**D**) 2-PLA, (**E**) 1-PLA or (**F**) Fab-PLA. Nuclei were stained with DAPI and presented as blue signals while the PLA signals are presented as red color dots. Scale bar: 5 μm. (**G**) Quantification of the results of IgM:IgM BCR proximity for resting (R) and NIP-BSA-stimulated (S) TKO-MD cells analyzed by 2-PLA, 1-PLA and Fab-PLA. For each experiment, the PLA signals (counts per cell) of each sample were counted from a minimum of 50 cells and then normalized to the PLA signal of the resting cells with Fab-PLA (set to 100%). Data represent the mean and SEM of five independent experiments. Significant difference between samples is highlighted by stars. (**H** and **I**) Representative confocal microscopic images (**H**) and quantified results (**I**) of IgM:IgM Fab-PLA of resting and NIP-BSA stimulated TKO-MD cells using different concentrations of Fab reagents. Data represent the mean and SEM of a minimum of three independent experiments. For each experiment, the PLA signals (counts per cell) of each sample were counted from a minimum of 1000 cells and were then normalized to the PLA signal of the resting B cells probed with the 1/100 Fab dilution. (**J**) Schematic drawing showing the spatial organization of the BCR as monitored by Fab-PLA on resting and stimulated B cells (left and middle panel) or 1-PLA on stimulated B cells (right panel).

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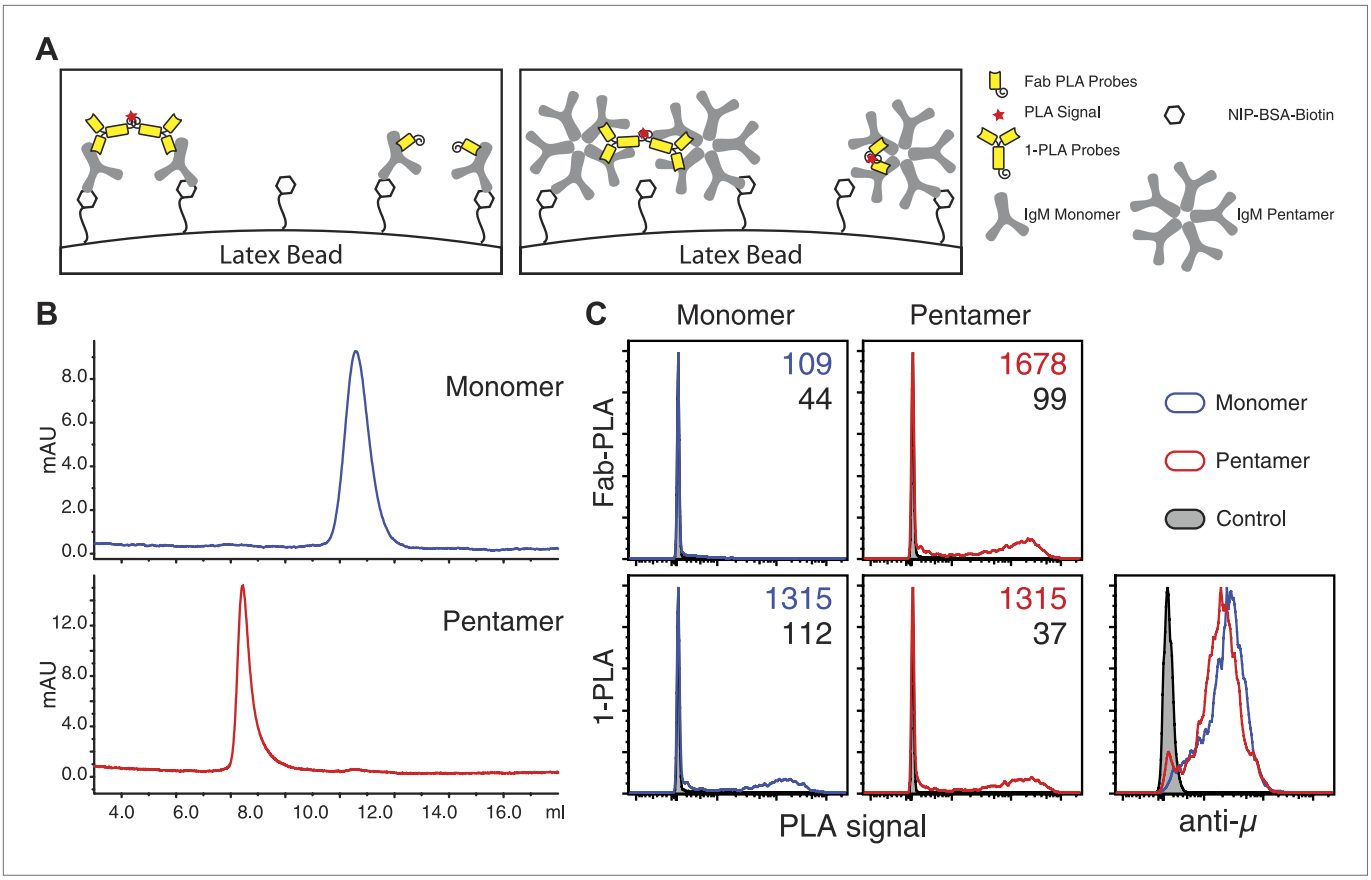

Figure 2. Fab-PLA but not 1-PLA is able to distinguish the oligomeric and monomeric BCR. (**A**) Scheme of the experiment setting. Latex beads coupled with low density of NIP-BSA were loaded with either the monomer or pentamer form of NIP-specific IgM antibodies. Due to the theoretical difference in their detection range (***Figure 1J***), 1-PLA is expected to obtain positive signals for beads loaded with either the monomeric or the pentameric IgM, while Fab-PLA is expected to show positive only for beads bound with pentameric IgM. (**B**) The size and purity of monomeric and pentameric IgM preparations were verified by size exclusion chromatography. (**C**) Comparison of beads loaded with monomeric (left) or pentameric (right) IgM measured by FACS after Fab-PLA (up) or 1-PLA (low) assay. Results were gated for the beads based on SSC and FSC. MFI of the PLA signals for the beads are marked on the upper right corner of each plot. The amount of IgM bound to the beads were monitored by anti-μ staining and shown at the lower right corner. Data are representative of three independent experiments.

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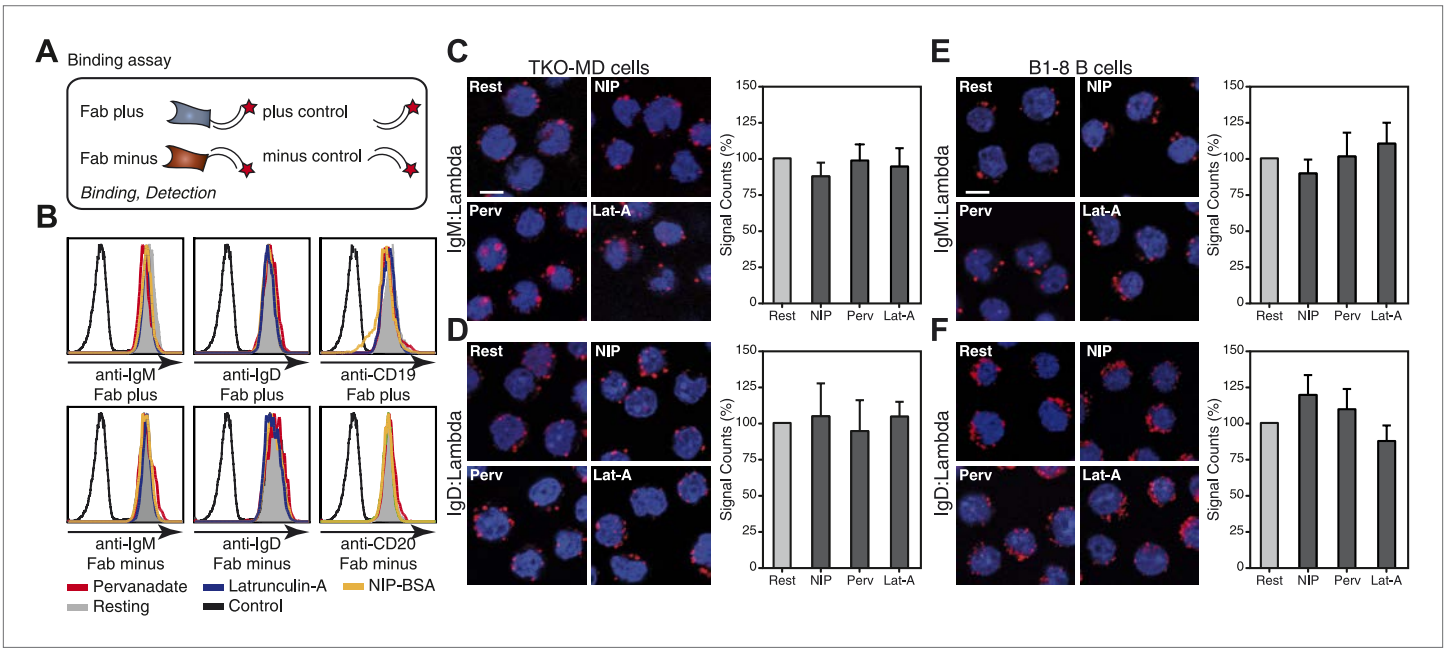

Figure 2—figure supplement 1. Similar binding efficiency of Fab-PLA probes on resting and stimulated B cells.

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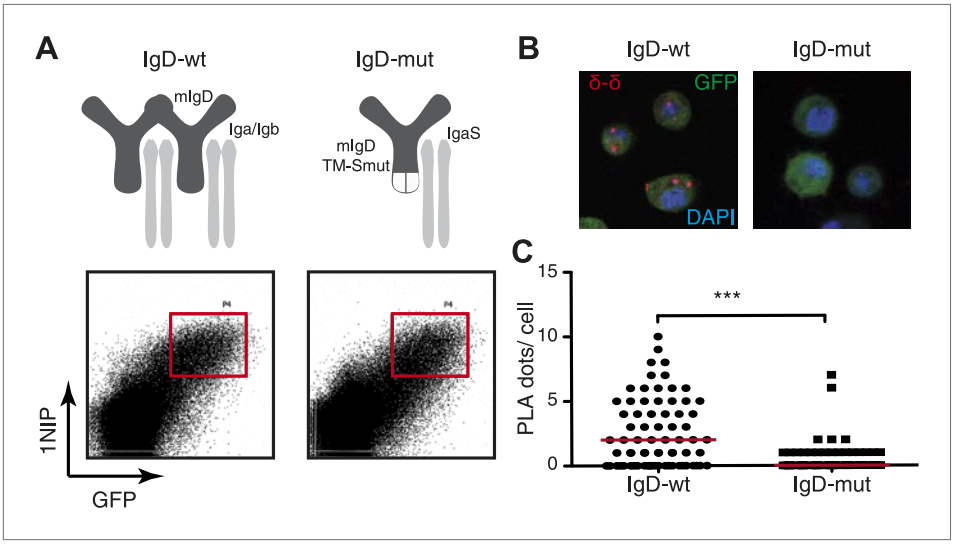

Figure 3. Fab-PLA detects IgD-BCR oligomers on the S2 cell surface. (**A**) Schematic drawing of wild type and double-mutant IgD-BCRs (δm transmembrane mutations and removal of the S–S bridge of the Igα/Igβ heterodimer) and their expression on transfected S2 cells analyzed by FACScan with a fluorescent 1NIP-peptide. Positively transfected S2 cells are indicated by the expression of a cotransfected GFP vector. The double-positive (GFP+, BCR+) S2 cell population, indicated by red square, were sorted and used for Fab-PLA. (**B**) Confocal microscopy analysis of the IgD:IgD Fab-PLA reaction of GFP+ S2 cells expressing wild type (left panel) or double-mutant (right panel) IgD-BCR. PLA signals are shown as red dots and nuclei were visualized by DAPI staining (blue). Scale bar: 5 μm. (**C**) Quantification of IgD:IgD Fab-PLA (each dot represents the amounts of Fab-PLA signals per S2 cell). The data were analyzed by the mann-whitney test and the median values are shown as red line.

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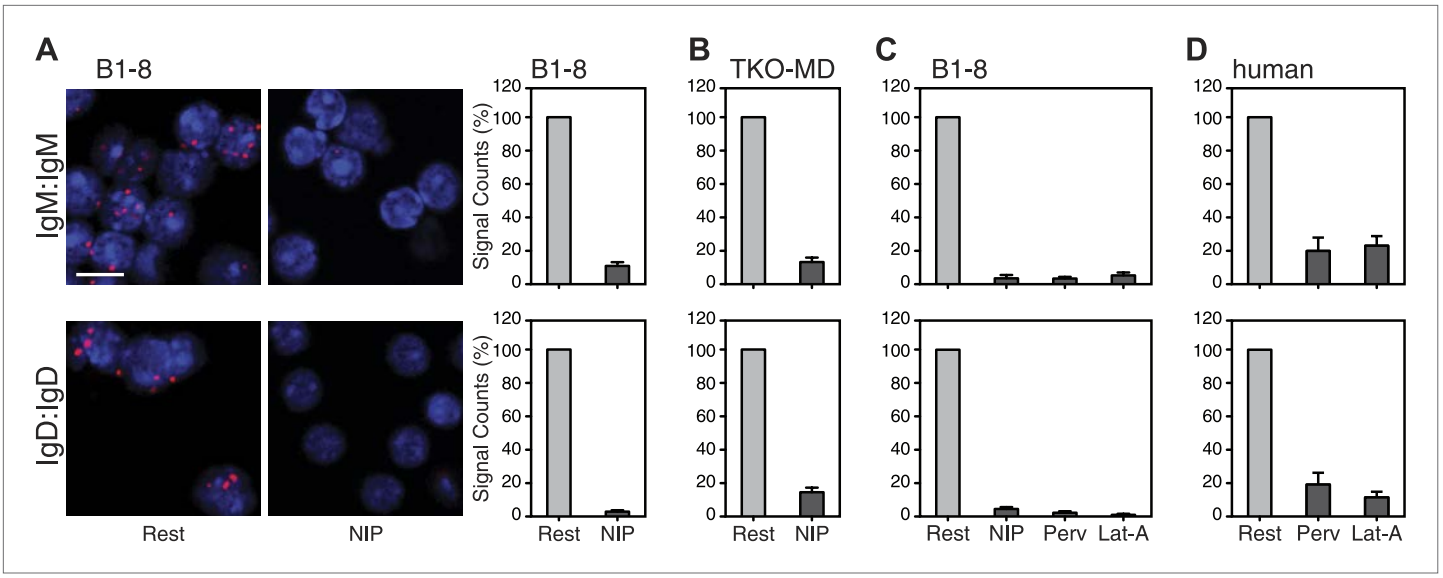

Figure 4. Fab-PLA study of the nanoscale organization of the IgM-BCR and the IgD-BCR on resting and activated B cells. (**A**) The IgM:IgM (upper) and IgD:IgD (lower) proximity of antigen receptors on resting or activated B1-8 splenic B cells were examined by Fab-PLA and shown as representative microscopic images (left) and quantified results (right). (**B**–**D**) Quantified Fab-PLA results indicate the IgM:IgM (upper) and IgD:IgD (lower) proximity on resting or activated TKO-MD cells (**B**), B1-8 splenic B cells (**C**) and human IgM+IgD+ naïve B cells isolated from peripheral blood (**D**). Scale bar: 5 μm. Quantified data represent the mean and SEM of a minimum of four independent experiments. For each experiment, PLA signals (counts per cell) of each sample were counted from a minimum of 100 cells and were then normalized to the PLA signals of the resting cells.

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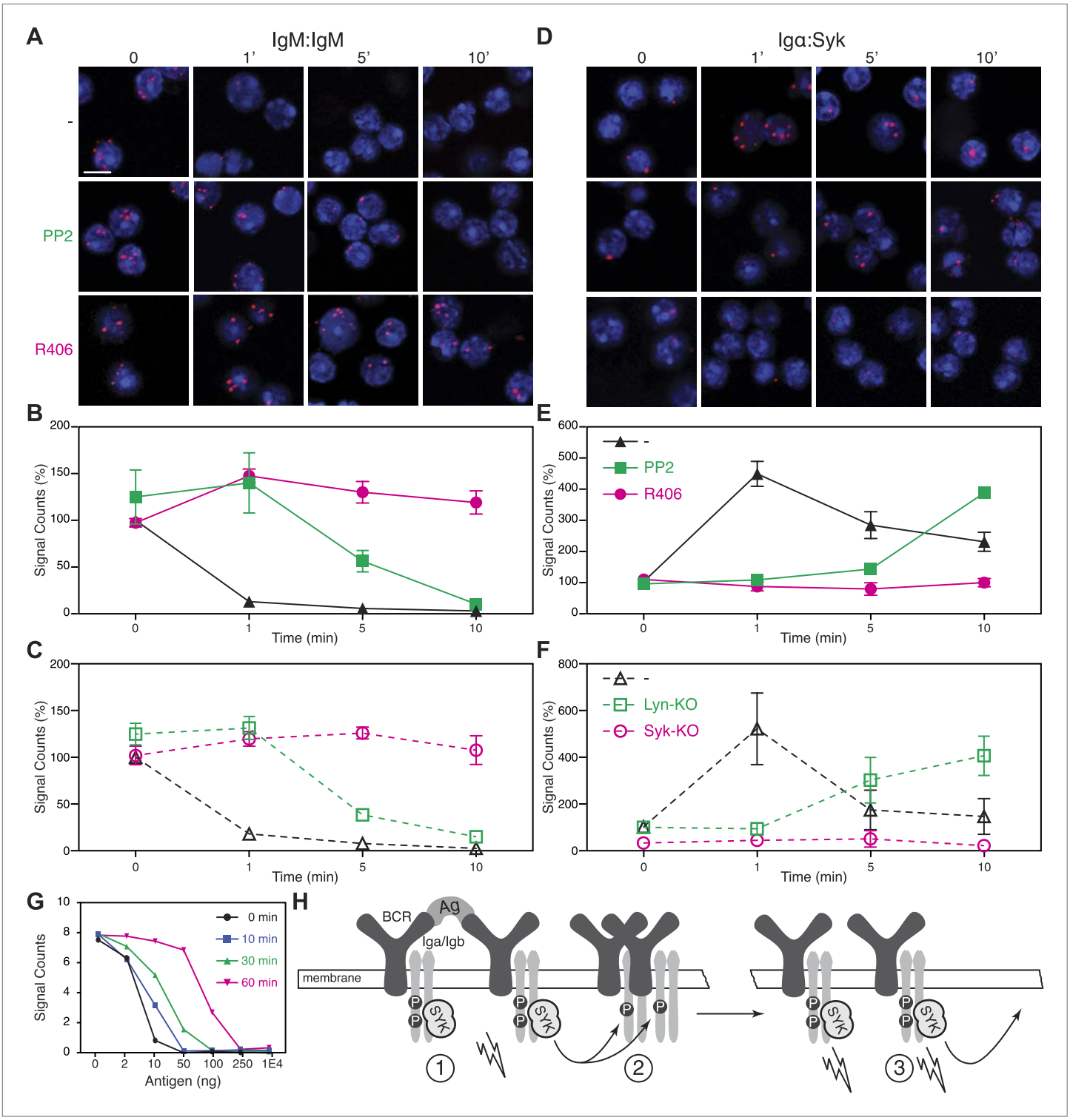

Figure 5. Syk activity is required for an efficient IgM-BCR dissociation after B cell activation. Representative microscopic images of the IgM:IgM (**A**) and the Igα:Syk (**D**) proximity detected by Fab-PLA. B1-8 B cells were analyzed at different time points (0, 1, 5, 10 min) after their activation with Lat-A in the absence (upper panels) or presence of the src-family kinase inhibitor PP2 (middle panels) or the Syk inhibitor R406 (bottom panels). Scale bar: 5 μm. Quantification of the IgM:IgM (**B**) and the Igα:Syk (**E**) Fab-PLA plotted as mean and SEM of a minimum of three independent experiments. For each experiment, PLA signals (counts per cell) of each sample were counted from a minimum of 500 cells and were then normalized to the PLA signals of resting untreated B1-8 B cells. Quantification of the IgM:IgM (**C**) and the Igα:Syk (**F**) proximity detected by Fab-PLA. B cells isolated from spleens of wild type, Lyn-KO and inducible, B cell-specific Syk-KO mice were analyzed at different time points (0, 1, 5, 10 min) after their activation with Lat-A. For each

Figure 5. Continued on next page

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experiment, PLA signals (counts per cell) of each sample were counted from a minimum of 500 cells and were then normalized to the PLA signals of resting untreated wild type B cells. Data represent the mean and SEM of a minimum of three independent experiments. (**G**), Quantification of the IgM:IgM Fab-PLA of TKO-M cells stimulated for 5 min with the indicated amounts (2, 10, 50, 100, 250, 10000 ng/ml) of the antigen NIP-BSA at different times (0, 10, 30, 60 min) after the pretreatment of the B cells with the Syk inhibitor R406. PLA signals (signal counts) of each sample were counted from a minimum of 500 cells. Data are representative of three independent experiments. (**H**) Schematic drawing showing the spreading and amplification of the BCR/Syk signal by an outside-in and inside-out signaling mechanism. (1) Formation of the BCR/Syk seed complex by antigen-engaged BCRs; (2) ITAM phosphorylation of neighboring (unengaged) BCR complexes; (3) Binding of Syk to the phosphorylated ITAM opens the receptor from the inside (inside-out signaling) and results in further signal spreading.

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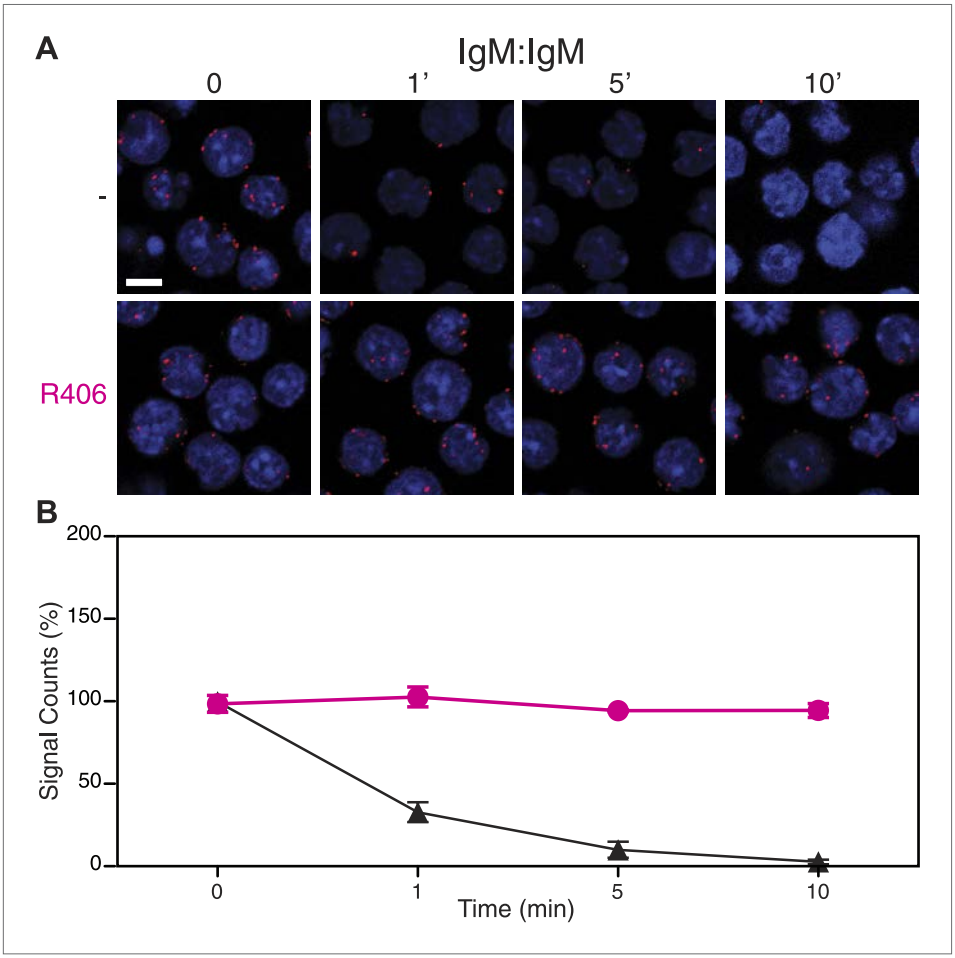

Figure 5—figure supplement 1. Syk activity is required for an efficient IgM-BCR dissociation on antigen stimulated B cells.

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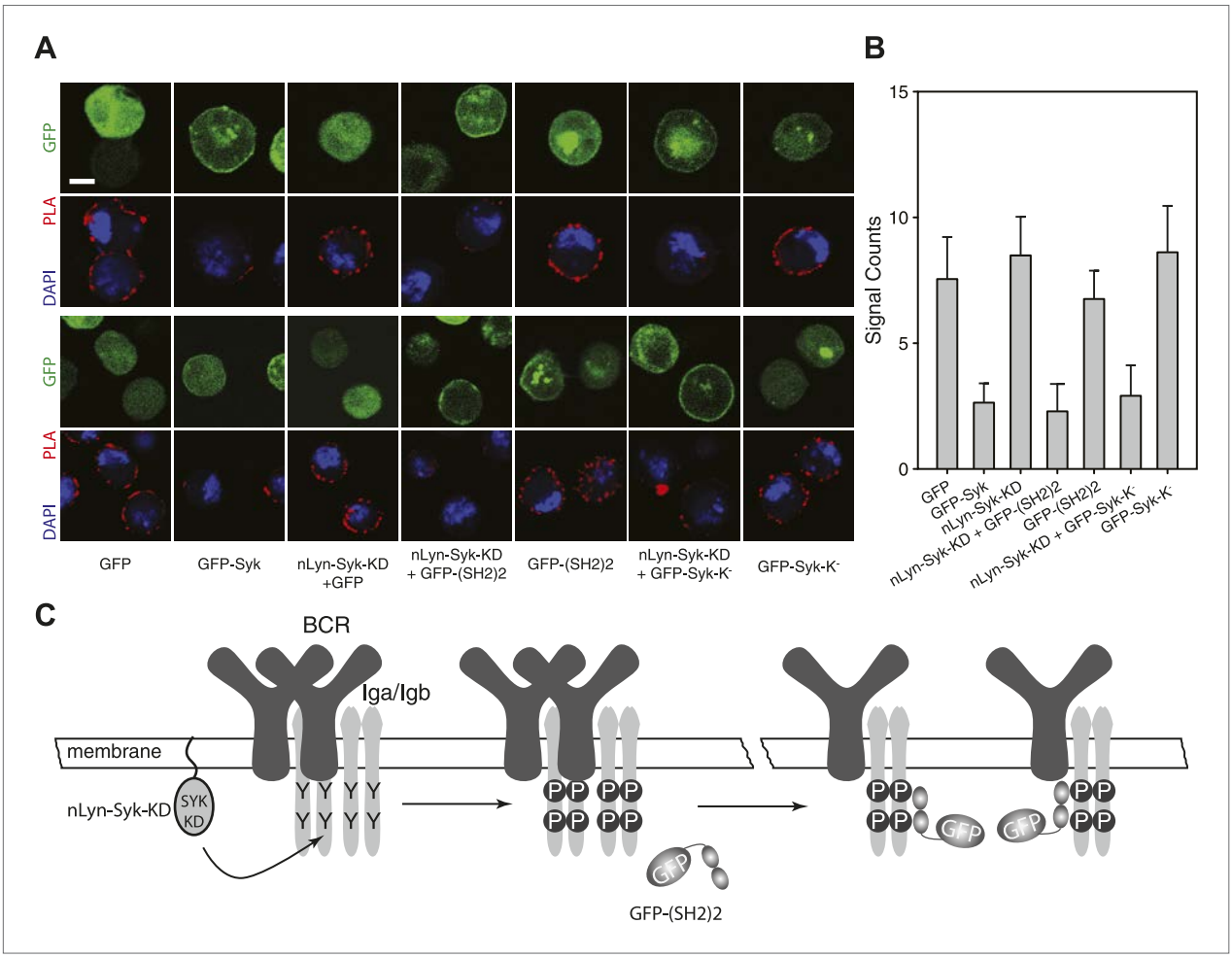

Figure 6. SH2 domains of Syk bind to phosphorylated ITAM and open the BCR. Representative microscopic images (**A**) and quantified results (**B**) show the IgM:IgM proximity of S2 cells expressing IgM-BCR together with the indicated constructs. GFP is shown as green, nuclei are stained with DAPI and shown as blue signals. The PLA signal is indicated by the red color. Scale bar: 5 μm. For the quantification, PLA signals (signal counts) of each sample were counted from a minimum of 500 cells. (**C**) Schematic drawing showing that not the ITAM phosphorylation tyrosines but the binding of the two tandem SH2 domains of Syk to the phosphorylated ITAM tyrosines is opening the BCR.

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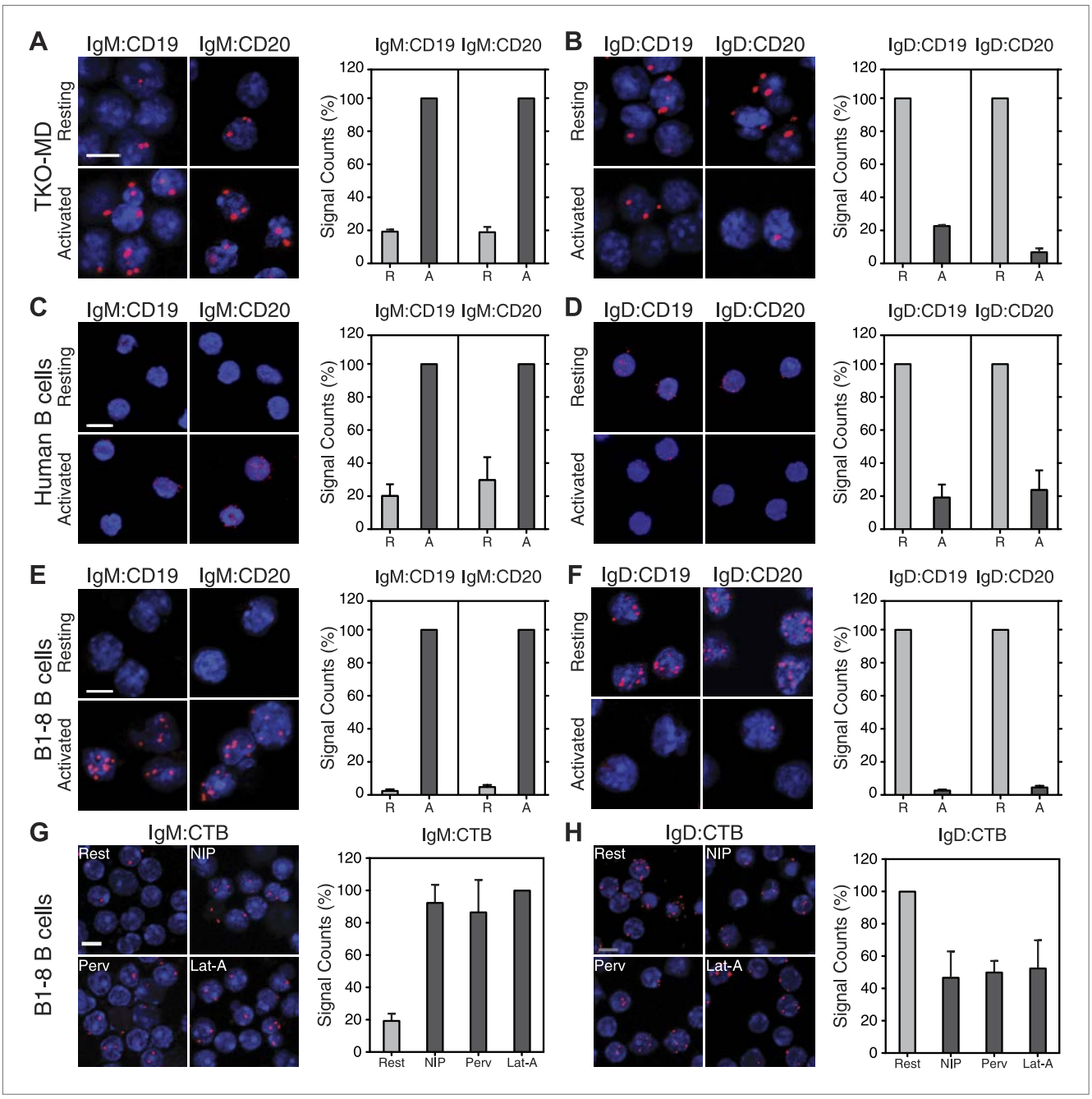

Figure 7. Fab-PLA of the nanoscale proximities of the IgM-BCR and IgD-BCR to the coreceptor molecules CD19/CD20 and the GM-1 Ganglioside. Representative microscopic images (left) and quantified results (right) of the proximity of CD19 or CD20 to the IgM-BCR (**A**, **C**, **E**) or the IgD-BCR(**B**, **D**, **F**) on resting or activated (**A** and **B**) TKO-MD, (**C** and **D**) human B cells and (**E** and **F**) B1-8 B cells stimulated for 5 min with NIP-BSA. (**G** and **H**) The proximity of the GM-1 Ganglioside (detected by CTB) to (**G**) the IgM-BCR or (**H**) the IgD-BCR on resting or activated B1-8 B cells stimulated for 5 min with the antigen NIP-BSA, the oxidant pervanadate and the F-actin inhibitor Lat-A, are shown as representative microscopic images (left) and quantified results (right). Scale bar: 5 μm. For the quantified results, data represent the mean and SEM of a minimum of three independent experiments. For each experiment, PLA signals (counts per cell) of each sample were counted from a minimum of 250 cells and were then normalized to the PLA signals of either (in **A**, **C**, **E**) the activated, or (in **B**, **D**, **F**, **H**) the resting B cells, or in (**G**) the Lat-A activated cells.

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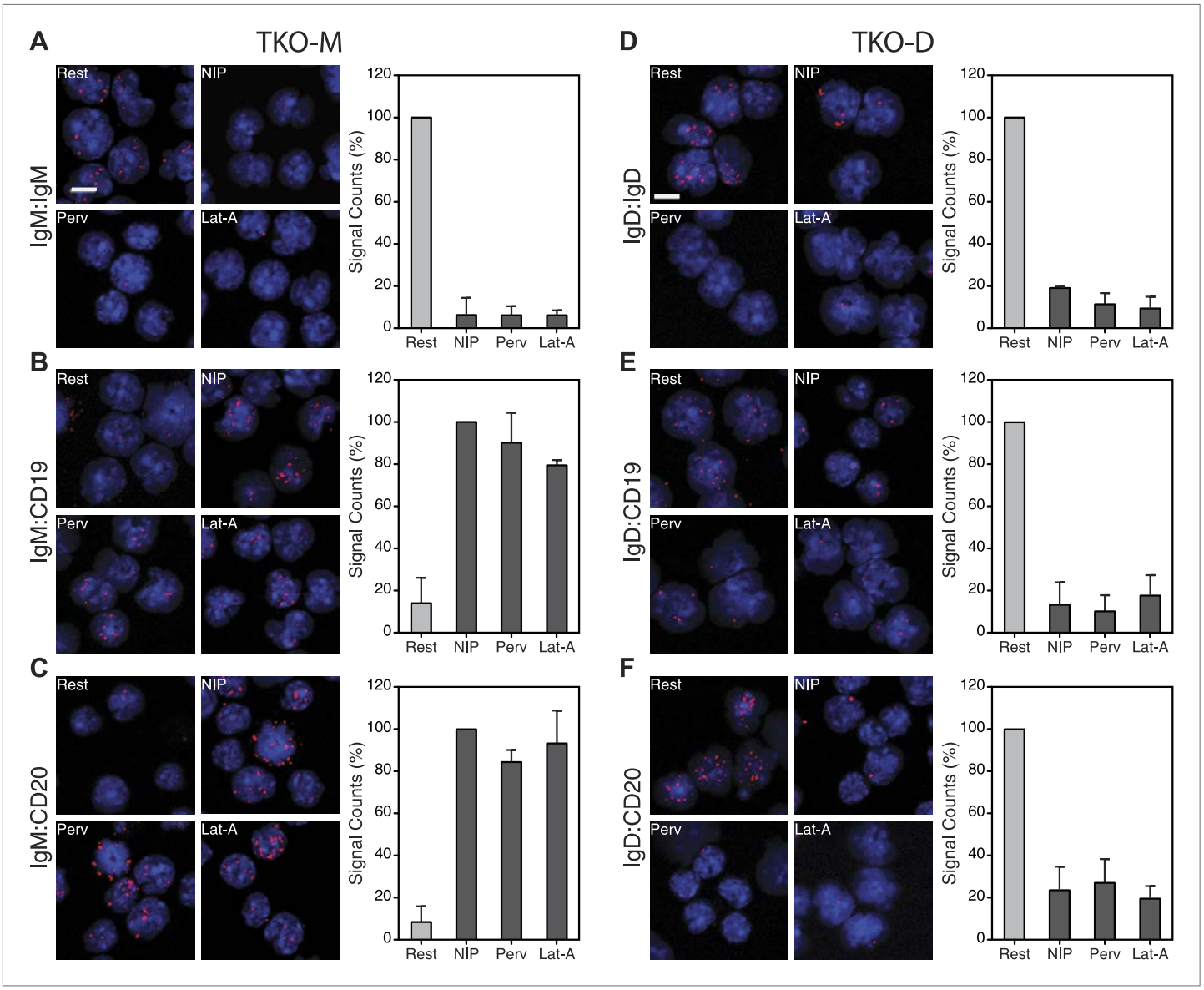

Figure 7—figure supplement 1. Nanoscale IgM-BCR and IgD-BCR dissociation and coreceptor reorganization on B cells expressing only one BCR isotype.

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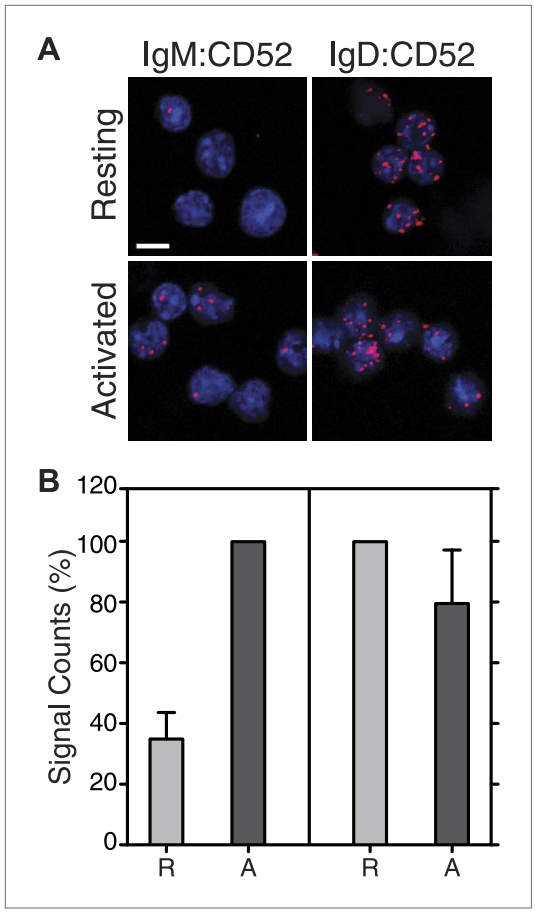

Figure 7—figure supplement 2. Fab-PLA of the nanoscale proximities of the IgM-BCR and IgD-BCR to the lipid raft marker CD52.

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