Supplementary materials for this manuscript include the following:

7 figures

4 videos

Figure 1-S1

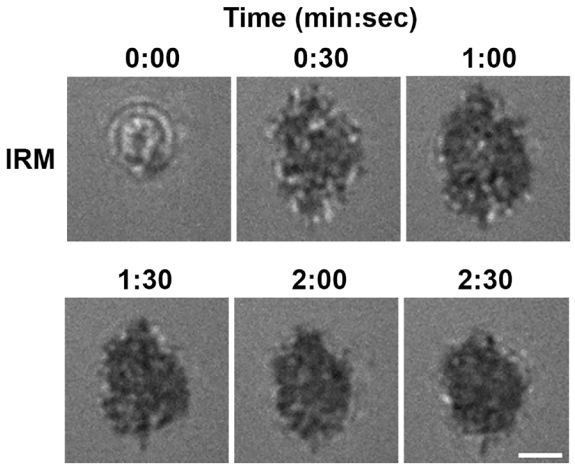
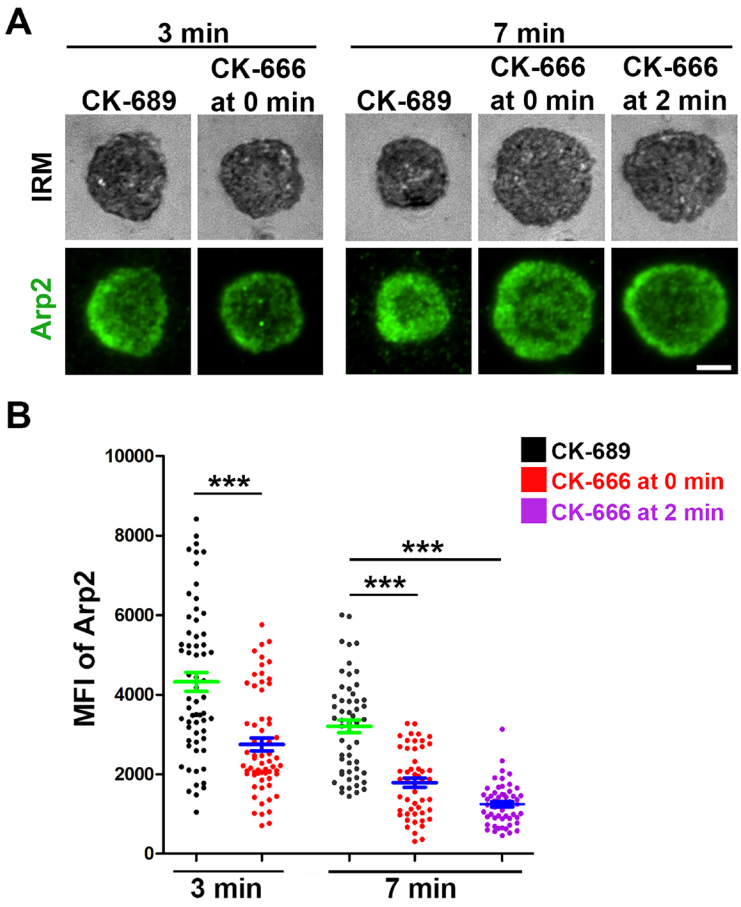


Figure 1-figure supplement 1. B-cells spread and contract on Fab'-coated-planar lipid bilayers. Splenic B-cells were pre-warmed to 37°C and incubated with planar lipid bilayers coated with monobiotinylated Fab' fragment of goat anti-mouse IgG+M (Fab'-PLB) and imaged live at 37°C by interference reflection microscopy (IRM). Shown are individual frames from a time-lapse IRM image of one B-cell. The plasma membrane area contacting with Fab'-PLB (B-cell contact zone) visualized by IRM increased between 0-1 min after landing, indicating spreading, and decreased after 1 min 30 sec, indicating contraction. Scale bar, 2 μm.

Figure 1-S2



1397

1398 **Figure 1-figure supplement 2. CK-666 significantly decreases Arp2/3 recruitment to the B-**

1399 **cell contact zone.** WT splenic B-cells were treated with CK-689 or CK-666 (50 μM) before (0

1400 min) and after maximal spreading (2 min) during incubation with Fab'-PLB at 37°C. Cells were

1401 fixed at 3 and 7 min, permeabilized, stained for Arp2, and imaged using IRM and TIRF. Shown

1402 are representative images (**A**) and the MFI of Arp2 in the contact zone at 3 min and 7 min

1403 compared between B-cell treated with CK-689 (black dots), CK-666 from 0 min (red dots), and

1404 CK-666 from 2 min (purple dots) (**B**). Data points represent individual cells from 3 independent

1405 experiments with ~20 cells per condition per experiment. Scale bar, 2 μm. *** $p<0.001$, by non-

1406 parametric student's *t*-test.

Figure 1-S3

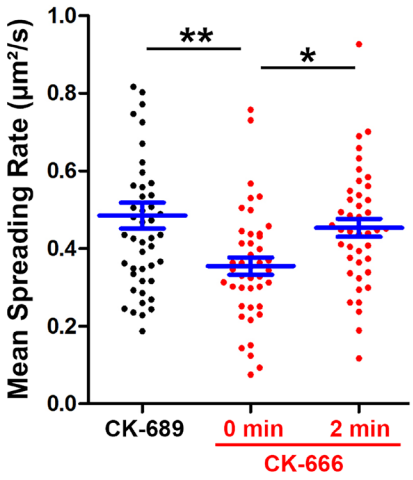
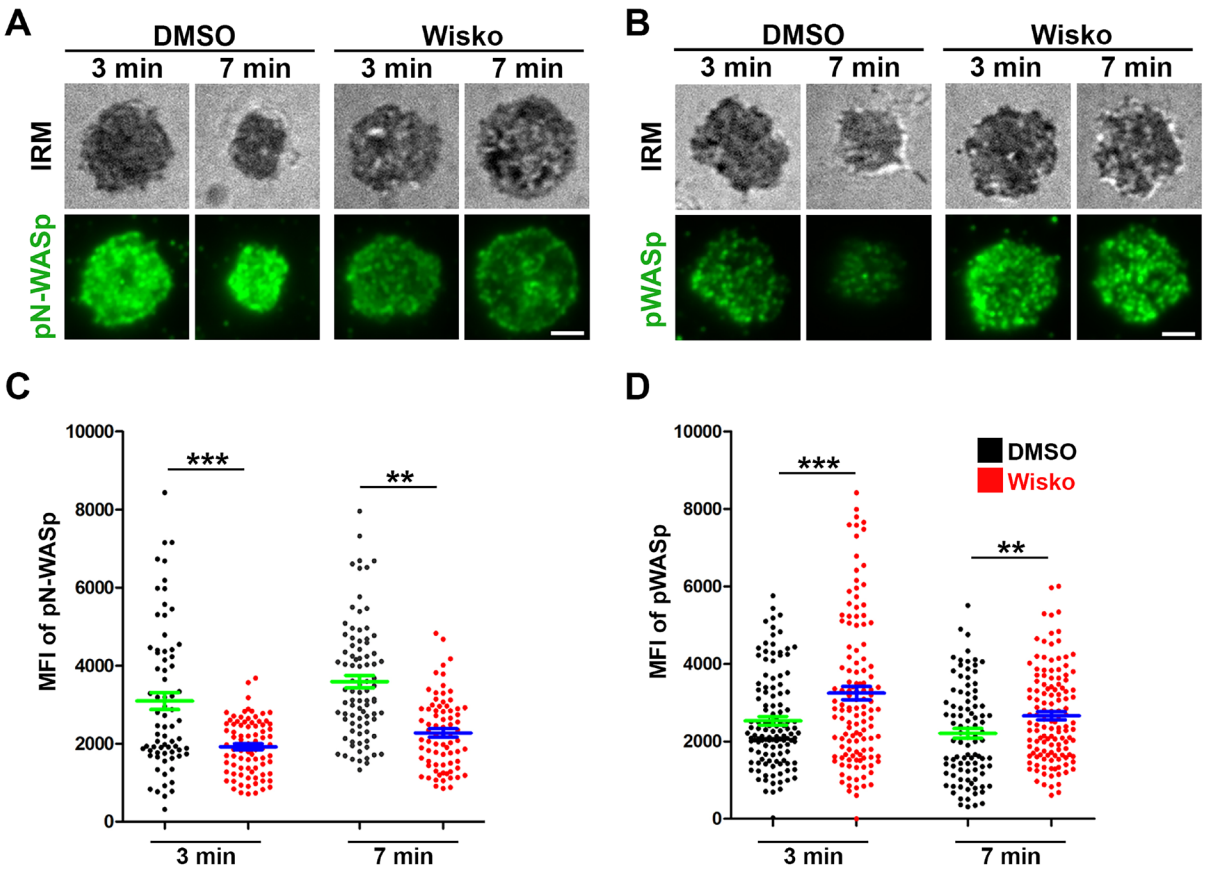


Figure 1-figure supplement 3. CK-666 treatment before but not after maximal B-cell spreading decreased the spreading kinetics. WT splenic B-cells were treated with CK-689 or CK-666 (50 μM) before (0 min) and after maximal spreading (2 min) during incubation with Fab'-PLB and imaged live at 37°C by IRM. The area occupied by the B-cell contact zone was measured using IRM images and custom codes made in MATLAB. The mean spreading rate of each cell during its early spreading phase was quantified using the contact area versus the time curve of that cell by linear regression. The averaged spreading rates (±SEM) were generated from 3 independent experiments with ~15 cells per condition per experiment. * $p>0.05$, ** $p<0.01$, by non-parametric student's *t*-test.

Figure 1-S4



1417

1418 **Figure 1-figure supplement 4. Wiskostatin treatment inhibits N-WASP activation while**

1419 **enhancing WASP activation in B-cells.** WT splenic B-cells were pre-treated with Wiskostatin

1420 (Wisko, 10 μM) or DMSO (control) for 10 min at 37°C before and during incubation with Fab'-

1421 PLB. Cells were fixed at 3 and 7 min, permeabilized, stained for phosphorylated N-WASP (pN-

1422 WASP) or WASP (pWASP), and imaged using IRM and TIRF. Shown are representative IRM

1423 and TIRF images of pN-WASP (**A**) and pWASP (**B**) at the B-cell contact zone and the MFI

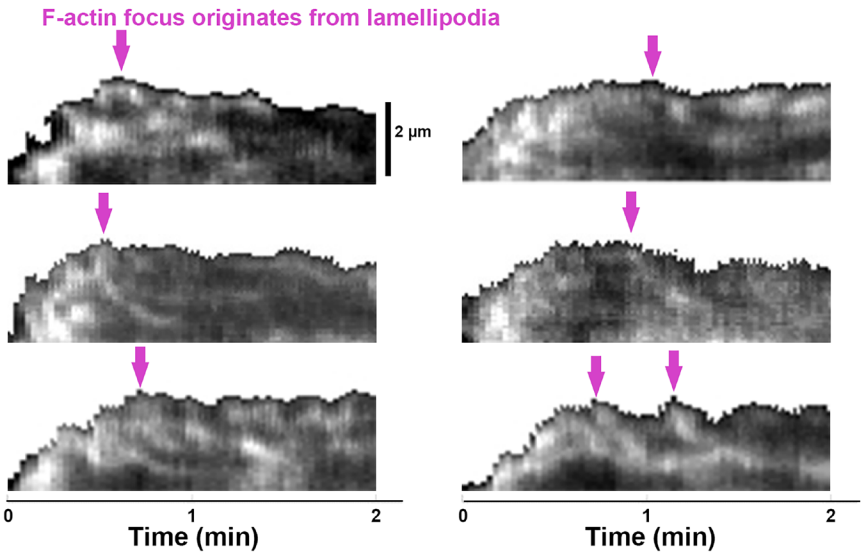
1424 (±SEM) of pN-WASP (**C**) and pWASP (**D**) in the B-cell contact zone, comparing between DMSO

1425 and Wisko-treated B-cells. Data points represent individual cells from 3 independent

1426 experiments with ~25 cells per condition per experiment. Scale bar, 2 μm. ** p<0.01, ***

1427 p<0.001, by non-parametric student's t-test.

Figure 3-S1



1428

1429 **Figure 3-figure supplement 1. Emerging of Inner F-actin foci from lamellipodia.** Splenic B-
1430 cells from LifeAct-GFP transgenic mice were treated with DMSO, imaged live using TIRF and
1431 IRM during incubation with Fab'-PLB at 37°C, and analyzed using kymographs that were
1432 randomly generated from each cell. Shown are six examples of the kymographs used for
1433 analysis. Arrows indicate the emergence of inner F-actin foci near the lamellipodia.
1434 Lamellipodia-derived inner F-actin foci were identified by their LifeAct-GFP FI ≥2 fold of their
1435 nearby region, inside location in the contact zone, migrating away the lamellipodial F-actin, and
1436 trackable for ≥8 sec.

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Figure 6-S1

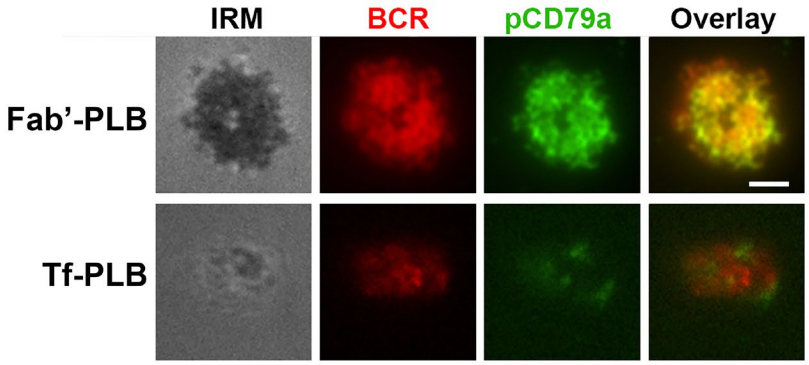

Figure 6-figure supplement 1. Fab'-PLB, but not Tf-PLB, induces BCR clustering and phosphorylation. WT splenic B-cells were pre-labeled with Cy3-Fab fragment of goat anti-mouse IgM+G at a concentration of 2.5 µg per 10^6 cells at 4°C for 30 min, followed by incubation with Fab'-PLBs or Tf-PLBs for 5 min at 37°C. Cells were fixed, permeabilized, stained for pCD79a, and imaged using IRM and TIRF. Shown are representative IRM and TIRF images from three independent experiments. Scale bar, 2 µm.

Figure 6-S2

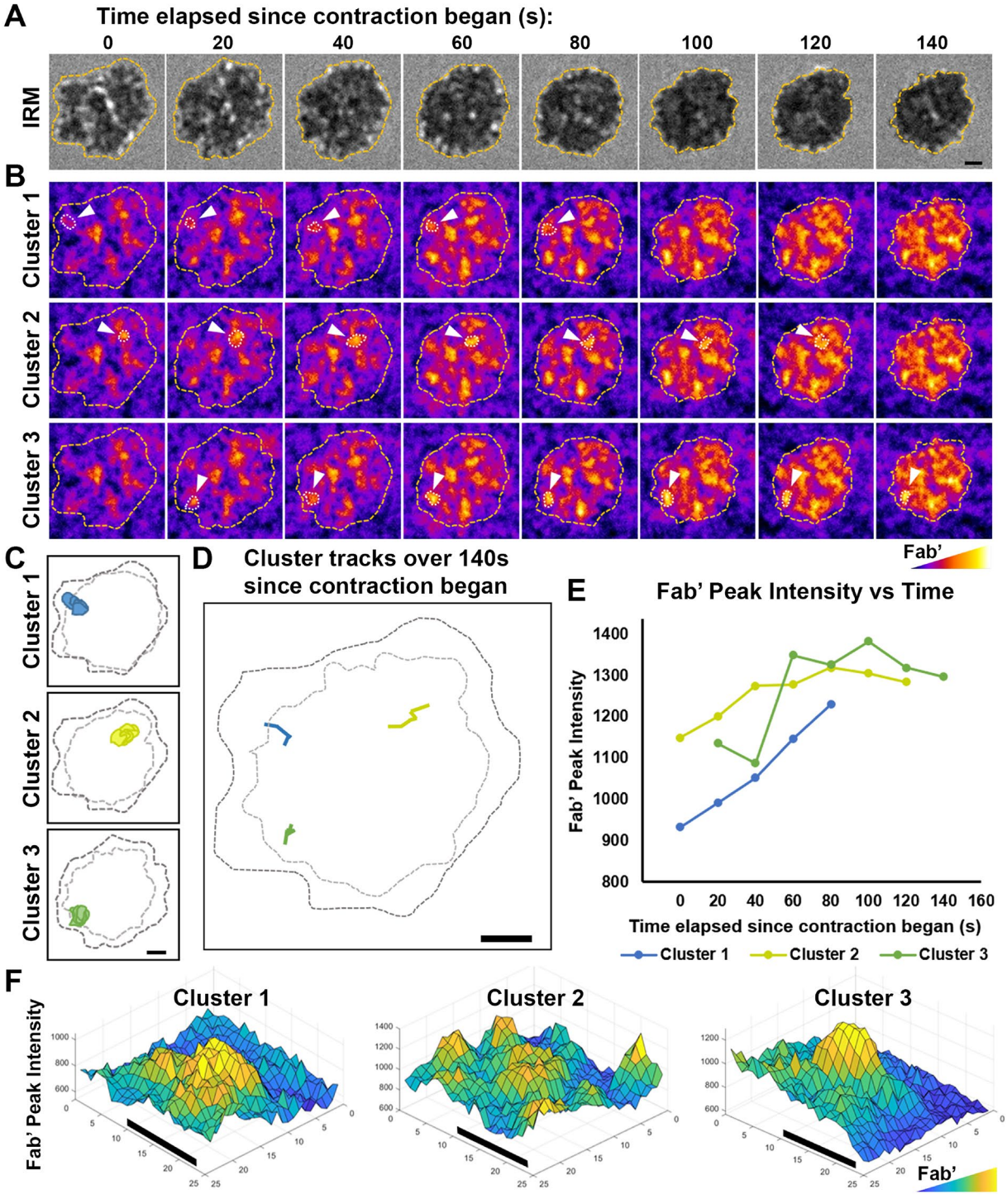

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Figure 6-figure supplement 2. Tracking and analyzing AF546-Fab' clusters in the B-cell contact zone. WT splenic B-cells were incubated with AF546-Fab'-PLB at 37°C and imaged live using IRM and TIRF. Shown are individual frames from time-lapse images of IRM (**A**) and TIRF (**B**), showing AF546-Fab' clusters within the contact zone of one DMSO-treated (vehicle control for Wisko) B-cell for 140 sec since the beginning of contraction. Fab' FI is shown as heat maps using NIH ImageJ. The boundary of the contact zone, detected using IRM images by a custom MATLAB script, is shown in yellow dashed lines. Arrows point to three representative clusters among the other clusters detected in the contact zone using custom MATLAB codes. Cluster detection masks for the three representative clusters are shown (**C**). Moving tracks for the three AF546-Fab' clusters are shown alongside the initial (black dashed lines) and final state (gray dashed lines) of the contact zone (**D**). Tracks were generated by following the peak of AF546 FI in each cluster as it moved. AF546-Fab' peak FI versus time curves for the three representative clusters are plotted over the duration that each cluster could be detected (**E**). Surface plots (2.5-D plots) of AF546-Fab' FI show a zoomed-in region consisting of each of the three AF546-Fab' clusters (**F**). Colors in (**B**) and (**F**) are scaled to AF546-Fab' FI values. Scale bars, 1 μm.

Video Legends

Figure 1-Video 1. Effects of CK-666, Wiskostatin, conditional N-WASP knockout, and WASP knockout on B-cell contraction. Splenic B-cells were incubated with planar lipid bilayers coated with monobiotinylated Fab' fragment of goat anti-mouse IgG+M (Fab'-PLB) in the absence and presence of various inhibitors and imaged live at 37°C at one frame per 2 seconds by interference reflection microscopy (IRM). Shown are representative IRM time-lapse images of WT B-cells treated with CK-689 or CK-666 (50 μM) before (0 min) and after maximal spreading (2 min) (**A**), WT B-cells treated with DMSO or Wiskostatin (Wisko, 10 μM) 10 min before and during incubation with Fab'-PLB (**B**), B-cells from flox control and B-cell-specific N-WASP knockout (cNKO) mice (**C**), and B-cells from WT or WASP knockout mice (WKO) (**D**). The frame in which the contact zone first appears was considered time 0. The videos are sped up by 20x compared to real time. Scale bar, 2 μm.

Figure 2-Video 1. Effects of CK-666 and WKO on F-actin foci formation. Splenic B-cells from LifeAct-GFP transgenic mice were incubated with Fab'-PLB in the absence and presence of CK-689 or CK-666 and imaged live at 37°C at one frame per 2 seconds by IRM and total internal reflection fluorescence microscopy (TIRF). Shown are representative IRM (**A** and **C**) and TIRF (**B** and **D**) time-lapse images of WT B-cells treated with CK-689 or CK-666 (50 μM) before and after maximal spreading (**A** and **B**) and WT and WKO B-cells expressing LifeAct-GFP (**C** and **D**). The video is sped up by 20x compared to real time. Scale bar, 2 μm.

Figure 5-Video 1. Wiskostatin treatment inhibits NMII ring-like structure formation. B-cells from mice expressing GFP fusion of non-muscle myosin IIA (GFP-NMIIA) and LifeAct-RFP transgenes were treated with DMSO or Wisko (10 μM) 10 min before and during incubation with Fab'-PLB. The B-cell contact zones were imaged live at one frame per 2 seconds using IRM

and TIRF. Shown are representative IRM and TIRF time-lapse images. The video is sped up by 20x compared to real time. Scale bar, 2 μm.

Figure 6-Video 1. Inhibition of B-cell contraction reduces the molecular density within BCR clusters. Splenic B-cells were incubated with AF546-Fab'-PLB in the absence and presence of various inhibitors and imaged live at 37°C at one frame per 2 seconds by IRM and TIRF. Shown are representative IRM **(A, C,** and **E)** and TIRF **(B, D,** and **F)** time-lapse images of WT B-cells treated with CK-689 or CK-666 (50 μM) before and after maximal spreading (**A** and **B**), WT B-cells treated with DMSO or Wisko (10 μM) 10 min before and during incubation with Fab'-PLB (**C** and **D**), and flox control or cNKO B-cells (**E** and **F**). TIRF images (**B, D,** and **F**) are shown as AF546-Fab' FI maps. The video is sped up by 20x compared to real time. Scale bar, 2 μm.