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Vinculin Arrests Motile B Cells by Stabilizing Integrin Clustering at the Immune Synapse

Julia Saez de Guinoa, Laura Barrio, and Yolanda R. Carrasco

Lymphocytes use integrin-based platforms to move and adhere firmly to the surface of other cells. The molecular mechanisms governing lymphocyte adhesion dynamics are however poorly understood. In this study, we show that in mouse B lymphocytes, the actin binding protein vinculin localizes to the ring-shaped integrin-rich domain of the immune synapse (IS); the assembly of this platform, triggered by cognate immune interactions, is needed for chemokine-mediated B cell motility arrest and leads to firm, long-lasting B cell adhesion to the APC. Vinculin is recruited early in IS formation, in parallel to a local phosphatidylinositol (4,5)-bisphosphate wave, and requires spleen tyrosine kinase activity. Lack of vinculin at the IS impairs firm adhesion, promoting, in turn, cell migration with Ag clustered at the uropod. Vinculin localization to the B cell contact area depends on actomyosin. These results identify vinculin as a major controller of integrin-mediated adhesion dynamics in B cells. The Journal of Immunology, 2013, 191: 2742–2751.

The regulated interplay between cell adhesion and cell motility is critical for B lymphocyte function. B cells must explore entire follicles in secondary lymphoid organs, where Ags are collected and presented by various APCs (1). To do this, B cells migrate continuously by random walking in response to the chemokine CXCL13 (2–4). This chemokine is produced mainly at the network of follicular dendritic cells; they expose it on their surface in the context of integrin ligands, which might assist in B cell motility (4, 5). Specific BCR recognition of Ag above a signaling threshold leads B cells to adhere firmly to the APC; a large LFA-1 integrin cluster is assembled, and the IS is formed (6, 7). The synapse platform has an important role in several aspects of the B cell activation process (6, 8, 9). The control of integrin activation, clustering, and localization thus underlies the precise modulation of B cell behavior.

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J.S.d.G. designed parts of the study, performed the experiments, analyzed the data, and assisted in manuscript preparation. L.B. assisted in performing experiments, data analysis, and manuscript preparation. Y.R.C. designed and supervised all aspects of the project and wrote the manuscript.

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Abbreviations used in this article: BAY, BAY 61-3560; Btk, Bruton’s tyrosine kinase; cSMAC, central supramolecular activation cluster; DIC, differential interference contrast; FA, focal adhesions; GPI, glycosylphosphatidylinositol; IRM, interference reflection microscopy; IS, immune synapse; NM-II, non–muscle motor protein myosin-II; PIP<sub>2</sub>, phosphatidylinositol (4,5)-bisphosphate; PIP<sub>2</sub>K<sub>1</sub>, type I phosphatidylinositol (4,5)-phosphate kinase; pSMAC, peripheral supramolecular activation cluster; RT, room temperature; sAg, soluble Ag; shRNA, short hairpin RNA; Syk, spleen tyrosine kinase; tAg, tethered Ag.

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Materials and Methods

Mice and cells

Primary B cells were freshly isolated from spleens of wild-type and MD4 BCR transgenic mice on the C57BL/6 genetic background by negative selection (>95% purity), as described (7). For time-lapse experiments, purified B cells were labeled before use with 0.1 µM CFSE long-term dye (Molecular Probes; 10 min, 37˚C). Animal experimentation was approved by the Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas Bioethics Committee and conforms to institutional, national, and European Union regulations. The A20 murine B cell line was transiently transfected by electroporation with PIPK1γ-GFP [a kind gift from Rosana Lacalle (27)], the PIP2 probe PLC6-PH-GFP [a kind gift from Isabel Mérida (28)], vinculin-GFP [a kind gift from Miguel Vicente-Manzaneares (29)], and the F-actin probe Lifeact-RFP constructs [a kind gift from Mario Mellado (30)] and used 20 h later for time-lapse experiments.

Time-lapse microscopy

We prepared artificial planar lipid bilayers containing glycosyl phosphatidylinositol (GPI)-linked mouse ICAM-1 (density 150 molecules/µm²) and when indicated, biotin-modified phospholipids at specific molecular densities (7). Membranes were assembled on FCS2 closed chambers (Biopetech) and blocked with PBS/2% FCS (1 h, room temperature [RT]). Ag (density 20 molecules/µm²) was tethered to the membranes by incubating with Alexa Fluor 647 or Alexa Fluor 555 streptavidin (Molecular Probes), followed by monobiotinylated hen egg lysozyme (Sigma-Aldrich) for wild-type B cells and the A20 B cell line. Before imaging, membranes were coated with 100 nM recombinant murine CXCL13 (PeproTech). We estimated the density of chemokine deposited on the lipid bilayer by an established immunofluorometric assay (6) using anti-mouse CXCL13 Ab (R&D Systems) and, for standard values, microbeads with calibrated IgG-binding capacities (Quantum Simply Cellular Kit, Bangs Laboratories); the value obtained was in the range of 30–40 molecules/µm². Unlabeled or CFSE-labeled primary B cells (2 × 10⁶) and transfected A20 B cells (1 × 10⁶) were injected into the warmed chamber (37˚C), and imaging was started. Confocal fluorescence, differential interference contrast (DIC), and interference reflection microscopy (IRM) images were acquired every 30 s for 20 min; when indicated, consecutive videos were acquired. Assays were performed in PBS/0.5% FCS/0.5 g l-glucose/2 mM MgCl₂/0.5 mM CaCl₂. For soluble Ag (sAg) stimulation, F(ab')₂ anti-IgM Ab (Jackson ImmunoResearch Laboratories) was added at 1 µg/ml final concentration to the B cell suspension immediately before injection into the FCS2 chamber. When indicated, primary B cells were treated with specified doses of the chemical inhibitor BAY 61-3606 (Bay, Calbiochem; 20 min, 30˚C) before injection. B cells were treated with blebbistatin (50 µM, Calbiochem) in situ by injection into the FCS2 chamber, incubated 5–10 min, and imaged. Images were acquired on an Axiovert LSM 510-META inverted microscope with a 40× oil immersion objective (Zeiss) and analyzed with Imaris 7.0 software (Bitplane).

Immunofluorescence

Primary B cells were in contact with planar lipid bilayers containing GPI-linked ICAM-1 and CXCL13 coating, alone or with tAg or with sAg (30 min), fixed with 4% paraformaldehyde (10 min, 37˚C), permeabilized with PBS/0.1% Triton X-100 (5 min, RT), blocked with PBS/2% FCS/2% BSA (overnight, 4˚C), and stained with Alexa Fluor 488 anti-IgM Ab (Jackson ImmunoResearch Laboratories) (30 min, RT). FCS2 chambers were imaged by confocal fluorescent microscopy on a Zeiss Axiovert inverted microscope (Zeiss) as above.

Imaging analysis

We used Imaris 7.0 software for qualitative and quantitative analysis of the image analysis. Sites at distinct cell plane and in the whole cell volume, as well as IRM area measurements. Ratios were obtained dividing the total fluorescence of the indicated protein at the synapse/contact plane between the total fluorescence at the midplane. Total fluorescence at the entire cell volume was obtained from z-stack images (optical slice thickness 1 µm) using Imaris 7.0 software.

Results

Infection using lentiviral vectors

Recombinant lentiviral particle stocks were obtained from HEK 293T cells by transfecting the short hairpin RNA (shRNA)-coding vector (pLKO.1, pGIPZ), the pMD.2G envelope vector, and the pCMV.R8.91 packaging vector (31). We used two types of shRNA coding vectors: mouse GIPZ lentiviral shRNAmir vectors (clones V2LMM_45006, V2LMM_56452, and V3LMM_437636, coding for mouse vinculin-specific shRNA, and a nonsilencing GIPZ lentiviral shRNAmir control; Thermo Scientific) and mouse vinculin shRNA in pLKO.1 vector backbones (clones NM_009502.3-3051_1, NM_009502.3-3154_1, and NM_009502.3-3147_1, coding for mouse vinculin-specific shRNA; Sigma-Aldrich). Briefly, 2 × 10⁶ cells were plated on p150 dishes (48 h) and then transfected with 2 µg envelope vector, 5 µg packaging vector, and 7 µg shRNA-coding vector by precomplexing with JetPEI (0.1 mM final concentration; PolyPlus Transfection; 30 min, RT) in OptiMEM (Life Technologies). After 4 h at 37˚C, we replaced medium with fresh DMEM/2% FCS; virus particles were harvested 48 or 72 h posttransfection. The virus suspension was filtered (23,000 rpm, 2 h, 4˚C). The pellet was resuspended in RPMI 1640 and stored at −80˚C. Freshly isolated primary B cells (2 × 10⁶) were infected with lentiviral particles (multiplicity of infection 1–10) in 500 µl RPMI 1640/10% FCS, alone or with recombinant murine IL-4 (30 ng/ml; PeproTech), CpG (1 µg/ml; InvivoGen), or LPS (2.5 µg/ml; Sigma-Aldrich) for 6 h at 37˚C. Medium was replaced with fresh RPMI 1640/10% FCS, alone or with the specified stimuli, and infected B cells cultured (48 h) to allow shRNA and gene reporter expression. Vinculin protein levels and GFP reporter expression were analyzed in Western blot.

Western blot analysis

Freshly isolated primary B cells (5 × 10⁵) were cultured in depletion medium (RPMI 1640/0.5% FCS; 1 h, 37˚C) and then stimulated with F(ab')₂ anti-IgM Ab (1 µg/ml; with shaking) or with ICAM-1/CXCL13 membranes in absence or presence of tAg for 30 min at 37˚C. Ice-cold PBS was added and B cells centrifuged (2000 rpm, 5 min, 4˚C) and lysed in RIPA lysis buffer with protease and phosphatase inhibitors (Roche; 30 min, 4˚C). Lysates were centrifuged (14,000 rpm, 30 min, 4˚C) and supernatants collected and stored at −80˚C. Lentiviral particle–infected B cell lysates were obtained similarly. Total protein was quantified with the Micro BCA Protein assay kit (Thermo Scientific), separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Bio-Rad). Blots were blocked with 2% BSA in TBST (10 mM Tris-HCL [pH 8], 150 mM NaCl, and 0.1% Tween-20) (1 h, RT) and incubated with rabbit anti-Syk, rabbit anti-phospho-Syk (Tyr352), rabbit anti–phospho-ERK1/2 (Tyr357/2), mouse anti–vinculin (clone hVIN-1; Sigma-Aldrich), mouse anti-β-actin (Sigma-Aldrich), or mouse anti–α-tubulin (clone DM1A; Sigma-Aldrich overnight, 4˚C), followed by HRP-conjugated secondary antibodies (AbD Serotec); the signal was detected with the ECL detection system (GE Healthcare). Signal intensity values in arbitrary units for each protein (p-Erk, vinculin) were quantified using ImageJ software (National Institutes of Health), normalized to tubulin or β-actin signal, relative to individual controls.

Statistical analysis

Graphs and statistical analysis were done using Prism 4.0 software (GraphPad). Two-tailed unpaired Student t test was applied: *p < 0.05, **p < 0.001, ***p < 0.0001.
and reproduces B cell dynamics to those observed in vivo by multiphoton microscopy (7).

Freshly isolated naive B cells from wild-type mouse spleen were labeled with the fluorescent probe CFSE and allowed to settle on artificial planar lipid bilayers containing GPI-linked ICAM-1 and a CXCL13 coating (ICAM-1/CXCL13 membranes), alone or with sAg (1 μg/ml F(ab’)2 anti-IgM Ab) or tAg (anti-κ L-chain at 20 molecules/μm²). We monitored B cell dynamics by time-lapse microscopy. B cells migrated by random walking across CXCL13 coated ICAM-1–containing artificial membranes (Supplemental Video 1). CXCL13 alone promoted LFA-1/ICAM-1 interactions (65%; detected by IRM; Fig. 1A) and a high frequency of cell polarization (70%), estimated by DIC as the fraction of cells with membrane protrusion activity (membrane ruffles) (Fig. 1B), as reported (7). Half of the polarized cells migrated across the substrate at a mean velocity of 4 μm/min (Fig. 1B, 1C, Supplemental Video 2). Although the presence of sAg increased the fraction of B cells with active LFA-1 (85% IRM⁺), it did not alter CXCL13-mediated cell polarization and migration. Motile B cells nonetheless showed a significant reduction in mean velocity, suggesting that sAg/BCR signaling impairs chemokine-mediated B cell motility (Fig. 1A, 1C). Recognition of tAg induced the highest frequencies of LFA-1–active B cells (95% IRM⁺); however, it completely abolished CXCL13-mediated B cell migration (Fig. 1A, 1C, Supplemental Video 3), as expected. We obtained similar results using MD4 BCR-transgenic B cells, their specific Ag hen egg lysozyme in membrane-tethered form and F(ab’)2 anti-IgM Ab as sAg (Supplemental Fig. 1A, 1B). These data thus indicated that tAg/BCR-mediated IS assembly is needed for B cell arrest, as LFA-1 activation triggered by sAg allowed motility.

Syk is important for integrin activation by the BCR (32) and by chemokine receptor stimulation (33). We compared the intensity of signals transmitted through the BCR after tAg and sAg stimulation by measuring p-Syk levels in B cells. Each stimulation condition led to distinct p-Syk patterns at the B cell–target membrane contact plane; with tAg, p-Syk concentrated at the IS cSMAC, whereas distribution was homogeneous in the case of sAg (Fig. 1D). Comparison of p-Syk fluorescence intensity at the B cell contact plane and the midplane showed polarization at the contact plane only in tAg conditions (Fig. 1D). p-Syk values at the B cell contact plane were significantly higher in presence of tAg than with sAg; results were similar for p-Syk quantified in the entire B cell volume (Fig. 1E, 1F). Syk protein levels were comparable in all conditions analyzed (Supplemental Fig. 1C). Greater Syk activation and localization at the B cell contact plane promoted by tAg/BCR signaling might be important for LFA-1 activation, synapse assembly, and B cell arrest.

Vinculin is recruited to the integrin-rich domain of the B cell IS

To determine the role of vinculin in stabilizing the B cell IS and arresting B cell motility, we analyzed vinculin at the B cell synapse. B cells in contact with ICAM-1/CXCL13 membranes with tAg (20 min) were fixed and stained for talin, vinculin, and F-actin. We detected vinculin at the B cell IS; it accumulated markedly in the ring-shaped pSMAC structure that matched the F-actin–rich domain (Fig. 2A). Its binding partner talin was also found at the IS, which colocalized mainly with the F-actin–rich pSMAC, but also

\[ \text{FIGURE 1.} \text{ tAg/BCR-triggered synapse assembly is necessary to arrest B cell motility. Primary wild-type B cells were allowed to settle on membranes containing GPI-ICAM-1 and coated with CXCL13, alone or with membrane-tAg or sAg. Frequency of cell adhesion (estimated by IRM) (A) and cell polarization and motility (estimated by DIC microscopy) (B) in the specified stimulation conditions. Data from a representative experiment are shown (n = 3). (C) Mean velocity values of motile B cells in (B); each dot represents a single cell. (D) DIC and fluorescence images for p-Syk (Alexa Fluor 488), F-actin (Alexa Fluor 647), and Ag (Alexa Fluor 555) at the contact plane and for p-Syk at the midplane of representative B cells in contact with ICAM-1/CXCL13 membranes with tAg or sAg. Profiles of relative mean fluorescence distribution of p-Syk, F-actin, and Ag at the contact plane (arrow in the merge image) and profiles of p-Syk fluorescence distribution at contact and midplanes (arrows in merge and middle plane images, respectively) in the presence of tAg or sAg (right panel). Total p-Syk fluorescence (FL) values at the contact plane (E) and in the entire cell volume (in arbitrary units [AU]) (F) of B cells settled on ICAM-1/CXCL13 membranes, alone (none) and with tAg or sAg; each dot represents a single cell. Data in (E) and (F) correspond to a representative experiment (n = 3). Scale bar, 2 μm. Statistical analysis, two-tailed unpaired Student t test was applied (Prism 4.0; GraphPad). *p < 0.05, ***p < 0.0001.} \]
in other parts of the contact area such as membrane ruffles (Fig. 2A). Comparison of fluorescent signals at the B cell contact plane with those at the midplane indicated that both vinculin and talin adaptor proteins were recruited to the IS. As we found that sAg/BCR signaling did not halt B cell motility, we tested the implication of vinculin in this observation by analyzing its localization to the contact site in sAg stimulation conditions. Vinculin did not localize nor was it distributed in a specific pattern at the plane of B cell contact with the target membrane; it accumulated mainly near the F-actin–rich cell edges (Fig. 2B). Quantification of vinculin at the contact area showed significantly lower values after BCR stimulation with sAg than with tAg; results were comparable when we analyzed total F-actin at the contact plane (arrow, merge image) and fluorescence distribution profiles for vinculin at contact and midcell planes (arrows in merge and midplane images, respectively) (right panel). (C) Total vinculin (left panel) and F-actin (right panel) fluorescence (FL) values in the B cell contact plane with the membrane alone (none) or with tAg or sAg (in arbitrary units [AU]); each dot represents a single cell. Data from a representative experiment are shown (n = 3). Scale bars, 2 μm. Statistical analysis, two-tailed unpaired Student t test was applied (Prism 4.0; GraphPad). ***p < 0.0001.

**FIGURE 2.** tAg/BCR signaling promotes vinculin polarization to the B cell synapse. Primary MD4 B cells in contact with ICAM-1/CXCL13 membranes with tAg or sAg were fixed and stained for the indicated markers. (A) DIC and fluorescence images are shown for vinculin (FITC), talin (FITC), F-actin (Alexa Fluor 647), and Ag (Alexa Fluor 555) at the contact plane and for vinculin and talin at the midplane of representative B cells in the presence of tAg. Relative mean fluorescence distribution profiles of vinculin, talin, F-actin, and Ag at the contact plane (arrow, merge image), and fluorescence distribution profiles of vinculin and talin at contact and midplanes (arrows in merge and midplane images, respectively) (right panel). (B) DIC and fluorescence images are shown for vinculin, F-actin, and Ag at the contact plane and for vinculin at the midplane of representative B cells in the presence of tAg or sAg. Relative mean fluorescence distribution profiles of vinculin, F-actin, and Ag at the contact plane (arrow, merge image) and fluorescence distribution profiles for vinculin at contact and midcell planes (arrows in merge and midplane images, respectively) (right panel). (C) Total vinculin (left panel) and F-actin (right panel) fluorescence (FL) values in the B cell contact plane with the membrane alone (none) or with tAg or sAg (in arbitrary units [AU]); each dot represents a single cell. Data from a representative experiment are shown (n = 3). Scale bars, 2 μm. Statistical analysis, two-tailed unpaired Student t test was applied (Prism 4.0; GraphPad). ***p < 0.0001.
the first 2.5 min, followed by formation of the ring structure in which it merged with vinculin (Supplemental Fig. 2, Supplemental Video 4).

Vinculin activation and translocation from cytosol to adhesion sites require its interaction with PIP$_2$ produced by PIPKI in other nonimmune cells. We studied PIPK$\gamma$ and PIP$_2$ dynamics at the B cell–target membrane contact plane by time-lapse confocal microscopy. We used a PIPK$\gamma$-GFP construct and the PIP$_2$ probe PLC$\gamma$-PH–GFP construct to transfect A20 B cells; transfectedants were tracked for synapse formation in contact with ICAM-1/CXCL13 membranes and tAg (anti-κ; 20 molecules/µm$^2$). We discarded those transfected cells showing high GFP levels for analysis. We

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** A wave of PIPK$\gamma$ and PIP$_2$ lipid is detected early in synapse formation. A20 B cells transfected with PIPK$\gamma$-GFP or PLC$\gamma$-PH–GFP (PIP$_2$ probe) constructs, in contact with ICAM-1/CXCL13 membranes and tAg, were monitored for synapse formation by time-lapse confocal microscopy. (A) Time-lapse DIC, IRM, and fluorescence PIPK$\gamma$ and Ag images of a representative B cell are shown. (B) Profiles of total PIPK$\gamma$ fluorescence (FL) values at the B cell–membrane interface plane over time, alone (none; bottom panel) and with tAg (top panel) of representative B cells; dashed line indicates initiation of B cell interaction with the membrane (detected by IRM; considered time zero). (C) Time-lapse DIC, IRM, and fluorescence PIP$_2$ and Ag images of a representative B cell. (D) As in (B), for the PIP$_2$ probe. (E) DIC, IRM, and fluorescence PIPK$\gamma$ and Ag images of a representative B cell. Relative mean fluorescence distribution profile of PIPK$\gamma$ and Ag at the contact plane (arrow, merge image) (bottom panel). (F) DIC, IRM, and fluorescence PIP$_2$ and Ag images of a representative B cell. Relative mean fluorescence distribution profile of PIP$_2$ and Ag at the contact plane (arrow, merge image) (bottom panel). Scale bars, 2 µm.
localized gradually to the synapse; the local PIP2 wave might be followed by tAg recognition; neither PIPKIγ nor marked changes in PIP2 levels were detected at the B cell–target membrane contact in the presence of the CXCL13 coating only (Fig. 3B, 3D). We also observed that PIPKIβ and PIP2 localization coincided with nascent LFA-1/ICAM-1 interactions (detected by IRM) and Ag clusters; with time, PIPKIγ persisted at the contact plane at lower levels, comparable to those in the rest of the B cell (Fig. 3A, 3B, Supplemental Video 5). PIP2 production followed a similar pattern, with a maximum immediately after the PIPKIγ peak early in synapse formation (Fig. 3C, 3D, Supplemental Video 6). Both PIPKIβ and PIP2 dynamics were triggered by tAg recognition; neither PIPKIγ recruitment nor marked changes in PIP2 levels were detected at the B cell–target membrane contact area in the presence of the CXCL13 coating only (Fig. 3B, 3D).

Then, after PIPKIγ produced the local PIP2 increase, vinculin localized gradually to the synapse; the local PIP2 wave might be needed for vinculin recruitment to the site of B cell–target membrane contact.

**Impaired vinculin recruitment to the synapse allows B cell motility**

To determine the relevance of vinculin in arresting motile B cells after tAg encounter, we attempted to knock down its expression in primary B cells. Lentiviral vectors coding for distinct mouse vinculin-specific shRNA were used to generate lentiviral particles and to infect B cells in several conditions (no stimulus, IL-4, CpG, and to infect B cells in several conditions (no stimulus, IL-4, CpG, or LPS; see Materials and Methods). At 48 h postinfection, we used Western blot to analyze vinculin levels in infected B cells. GFP reporter expression confirmed primary B cell infection by the lentiviral particles, with distinct efficiency depending on the stimulation (Supplemental Fig. 3A). We nonetheless found no clear reduction in vinculin levels with any of the shRNA used (Supplemental Fig. 3A, 3B). We did not analyze longer time points because infected primary B cells died or differentiated into plasma cells.

As vinculin recruitment was associated with strong local Syk activation through tAg/BCR signaling (Fig. 1D, 2B), we tested another approach to impair vinculin function. We used the specific chemical inhibitor BAY to interfere with Syk activity. B cells were treated with several BAY doses (1 to 0.1 μM); after BCR stimulation, we evaluated Syk activity by detection of p-ERK, a downstream effector. We also assessed the ability of BAY-treated B cells to migrate on ICAM-1/CXCL13 membranes to determine Syk inhibition downstream of CXCR5. High BAY doses (1 and 0.6 μM) abolished BCR-mediated Syk activation and reduced chemokine-triggered B cell motility (Supplemental Fig. 4A, 4B). Treatment with 0.3 μM BAY impaired BCR-triggered Syk activity, but allowed a higher frequency of migrating B cells, with no significant alteration in mean velocity compared with controls (Supplemental Fig. 4A, 4B). We evaluated the effect of this BAY dose on vinculin recruitment to the IS. BAY-treated B cells showed lower vinculin levels at the contact plane and a distribution pattern distinct from that of untreated B cells (Fig. 4A, Supplemental Fig. 4C). Absence of the F-actin–rich ring indicated profound alterations in pSMAC assembly in BAY-treated B cells (Fig. 4A). The ratio of vinculin at the contact plane with those at the midplane indicated almost no vinculin recruitment to the IS in BAY-treated B cells; F-actin polymerization was also impaired (Fig. 4B, Supplemental Fig. 4C). BAY treatment did not alter cSMAC formation or talin polarization to the contact plane (Fig. 4C). B cells treated with a lower BAY dose (0.1 μM) did not show any change in vinculin recruitment and localization at the IS (Supplemental Fig. 4C, 4D).

We used time-lapse microscopy to monitor the behavior of CFSE-labeled B cells, untreated or treated with 0.3 μM BAY, in

**FIGURE 4.** Syk activity inhibition impedes vinculin localization to the IS. Untreated or BAY-treated primary MD4 B cells in contact with ICAM-1/CXCL13 membranes and tAg. (A) DIC and fluorescence images of vinculin (FITC), F-actin (Alexa Fluor 647), and Ag (Alexa Fluor 555) at the synapse plane and of vinculin at the midplane of a representative B cell. (B) Ratio of total vinculin (left panel) and total F-actin (right panel) fluorescence (FL) at the synapse plane to that at the midplane for untreated and BAY-treated B cells; each dot represents a single cell. Data from a representative experiment are shown (n = 3). (C) DIC and fluorescence images of talin, F-actin, and Ag at the synapse plane of representative B cells. Ratio of total talin fluorescence at the synapse plane to that of the midplane for untreated and BAY-treated B cells (right panel); each dot represents a single cell. Data from a representative experiment (n = 4). Statistical analysis, two-tailed unpaired Student t test was applied (Prism 4.0; GraphPad). Scale bars, 2 μm. *p < 0.05, ***p < 0.0001.
contact with ICAM-1/CXCL13 membranes with tAg. Untreated B cells showed the predicted IS establishment and CXCL13-driven membrane ruffles; cells hardly moved from their position (Fig. 5A, Supplemental Video 7). A large percentage of BAY-treated B cells (40%) assembled an Ag cluster and migrated across the membrane (Fig. 5A, 5B, Supplemental Video 8). Motile BAY-treated B cells extended a clear lamellipodium at the cell front and carried the Ag cluster at the back uropod (Fig. 5A, Supplemental Video 8); they reached mean velocity values at ∼3 μm/min (Fig. 5C). These data indicated that vinculin recruitment is important for adhesion strength at the IS and to arrest chemokine-mediated B cell motility.

Non–muscle myosin-II activity is necessary for vinculin function at the B cell synapse

In nonimmune cells, vinculin recruitment to and function at focal adhesions requires non–muscle motor protein myosin-II (NM-II) activity (34). Active NM-II is present at the B cell synapse (7). We studied the role of NM-II in vinculin function at the B cell IS using the specific chemical inhibitor blebbistatin to interfere with NM-II activity. B cells were allowed to settle and establish an IS in contact with ICAM-1/CXCL13 membranes and tAg. After blebbistatin treatment (20 min), we fixed cells and stained for vinculin and F-actin. NM-II inhibition resulted in vinculin ring disorganization and loss of vinculin localization to the synapse (Fig. 6A, 6B). At the time analyzed, F-actin distribution was maintained surrounding the cSMAC, and its levels at the IS contact plane were lost (Fig. 6A, 6B). Before fixation, blebbistatin-treated B cells remained adhered to the membrane (detected by IRM), although the contact area was significantly reduced (Fig. 6C), suggesting internal disorganization of the pSMAC structure. Blebbistatin-treated B cells showed no sign of motility on the membranes, as full NM-II activity is needed for CXCL13-mediated B cell migration.

We used A20 B cells expressing a vinculin-GFP construct and Lifeact-RFP to monitor the effect of NM-II inhibition in time-lapse experiments. A20 B cells formed a mature IS in contact with ICAM-1/CXCL13 membranes and tAg; vinculin and F-actin distributed in a ring surrounding the cSMAC (Fig. 6D). After adding blebbistatin, we tracked the molecular dynamics of vinculin and F-actin at the IS by confocal microscopy. By 20 min posttreatment, the vinculin pattern was completely disorganized and its fluorescent signal declined; F-actin polymerization was reduced, and no longer confined to the vicinity of the cSMAC (Fig. 6D, Supplemental Video 9).

The stability of the vinculin-rich domain at the B cell IS thus depends on appropriate NM-II activity. The data also indicated that loss of vinculin is accompanied by diminished F-actin polymerization and F-actin ring disassembly at the contact plane.

Discussion

Our study showed that vinculin is recruited to the B cell IS and distributed in the LFA-1–rich pSMAC domain together with F-actin, talin, PIPKιγ, and the lipid PIP2. tAg/BCR-triggered Syk activity is needed for vinculin localization to the B cell–APC contact site; absence of vinculin recruitment allows B cells to continue moving in response to CXCL13 while assembling and carrying the synapse-characteristic Ag cluster at the uropod. Loss of vinculin also reduced F-actin polymerization, but not talin recruitment to the synapse. The motor protein NM-II is implicated in maintaining

**FIGURE 5.** Lack of vinculin at the synapse allows B cell motility. Untreated or BAY-treated primary B cells in contact with ICAM-1/CXCL13 membranes and tAg. (A) Time-lapse DIC and overlaid IRM/fluorescence Ag (Alexa Fluor 555) images of representative untreated (top panel) and BAY-treated (bottom panel) B cells. Arrows indicate Ag cluster position. (B) Migration frequency of untreated or BAY-treated B cells on ICAM-1/CXCL13 membranes, alone (empty bar) or with tAg (filled bars). (C) Mean velocity values of the motile B cells in (B); each dot represents a single cell. Data in (B) and (C) are the merge of three experiments. Scale bar, 2 μm. Statistical analysis, two-tailed unpaired Student t test was applied (Prism 4.0; GraphPad). **p < 0.001, ***p < 0.0001.
vinculin at the IS. These data identify vinculin as a key regulatory element of the assembly and stability of the LFA-1–mediated platforms that support B cell dynamics (i.e., synapse and kinapse).

Vinculin is found in the synapse of Jurkat T cells, in a complex containing WA VE-2, Arp2/3 and talin; vinculin is necessary for talin recruitment, but not for F-actin polymerization or integrin accumulation (35). Talin-deficient T cells do not adhere firmly to APC or arrest migration; they assemble some LFA-1 clustering, but did not recruit vinculin or F-actin to the short-lived synapse; the authors highlighted the importance of talin for T cell IS stability (36). In NK cells, LFA-1/ICAM-1 interaction leads to vinculin accumulation at the synapse; talin is needed for vinculin and F-actin localization to the contact site (37). In this study, we report that vinculin is recruited to the B cell synapse, specifically to the LFA-1–rich pSMAC domain, where it colocalizes with talin and F-actin. Talin might be also important for vinculin recruitment to the B cell synapse, a subject for further study. We nonetheless found that, without affecting talin localization, vinculin at the B cell synapse

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** NM-II activity regulates vinculin function at the synapse. Primary MD4 B cells in contact with ICAM-1/CXCL13 membranes and tAg were untreated or treated with blebbistatin (20 min), fixed, and stained for the indicated markers. (A) DIC and fluorescence images for vinculin (FITC), F-actin (Alexa Fluor 647), and Ag (Alexa Fluor 555) of representative untreated (control) and blebbistatin-treated B cells. Relative mean fluorescence (FL) distribution profiles of vinculin, F-actin, and Ag at the contact plane (arrow, merge image) (right panel). (B) Ratio of total vinculin (left panel) and total F-actin (right panel) fluorescence at the synapse plane to that at the midplane for untreated (control) and blebbistatin-treated B cells; each dot represents a single cell. Data from a representative experiment (n = 3). (C) DIC and IRM images of representative B cells, untreated, or treated with blebbistatin before fixing (left panel). B cell–membrane contact area values in the indicated conditions (right panel); each dot represents a single cell. Data correspond to the merge of two experiments. (D) A20 B cells cotransfected with vinculin-GFP and Lifeact-RFP constructs were allowed to form a synapse with ICAM-1/CXCL13 membranes and tAg, and blebbistatin was added. Time-lapse DIC, IRM and fluorescence vinculin, F-actin and Ag images of a representative A20 B cell before (preblebbistatin) and after blebbistatin addition. Scale bar, 2 μm. Statistical analysis, two-tailed unpaired Student t test was applied (Prism 4.0; GraphPad). ***p < 0.0001.
was essential for F-actin accumulation, pSMAC assembly and synapse stability. Although chemical inhibitors might have undesired effects, they were important tools to assess vinculin function in this study, due to the limitations we found to diminish vinculin expression in B cells by shRNA techniques and the nonviability of vinculin-deficient mouse models (22).

PIPKI localization and activity regulates the targeted, limited production of PIP2, which in turn governs the temporal and spatial requirements of adhesion site dynamics. In neutrophils, correct distribution of distinct PIPKI regulates cell polarity and migration (27, 38). PIPKιy deficiency at FA impairs talin and vinculin recruitment to nascent adhesion sites, which reduces integrin-mediated cell adhesion and force coupling (39). Another report does not implicate PIP2 in vinculin recruitment to adhesion sites, but rather in vinculin release and FA disassembly (40), which is reinforced by the finding that PIPKιy-deficient T cells show increased integrin-mediated adhesion (41). Our findings coincide with the first model; tAg/BCR stimulation promoted PIPKιy localization, and thus, local PIP2 production at the nascent synapse that could support vinculin recruitment. They remain detectable at the pSMAC of the mature synapse, possibly assisting the active vinculin conformation. PIPKιy is also found at the T cell synapse; the spatiotemporal regulation of PIP2 synthesis appears to control T cell rigidity and signaling organization (42).

FA are mechanosensitive structures that transmit cell forces to the extracellular matrix. Cell forces are generated as a consequence of NM-II action on the actin cytoskeleton (43). NM-II-mediated contractility controls the localization to FA of vinculin and other adaptor proteins (34). The ability of vinculin to bear force determines the assembly or disassembly of adhesion sites under tension (16, 17). In the podosome, another type of actomyosin-based integrin-rich platform, NM-II activity is not necessary for adaptor protein composition, which is controlled by the actin network (44). NM-II participates in synapse and migratory junctions in lymphocytes (7, 45–47). Our data show that NM-II activity is important for vinculin localization at the B cell synapse, as described for FA. Lack of vinculin due to NM-II inhibition led to reduced, mislocalized F-actin polymerization and pSMAC disassembly. We propose that vinculin is the mechanical sensor also at the lymphocyte synapse; it regulates assembly and disassembly of the adhesion structure based on cell force input. Two recent reports in T cells also highlight the relevance of NM-II–generated mechanical forces on modulating synapse assembly and cell activation (48, 49); both studies involved CasL, a member of the mechanosensing Cas protein family and predominantly expressed in T cells, in the mechanical sensing.

We found accumulation of active Syk (p-Syk) at the cSMAC of the mature B cell synapse. It has been previously showed some colocalization of p-Syk with the BCR-Ag central cluster at the mature synapse of naive B cells and localization of GFP-Syk at the cSMAC in A20 B cells by TIRFM (50). Although some reports in T cells showed that Ag receptor proximal signaling does not occur in the cSMAC (51, 52), it has been proposed that Ag quality (i.e., Ag affinity and abundance) determines the balance between signaling and receptor degradation at the cSMAC (53). The p-Syk enrichment at the cSMAC of the B cell IS detected in our study might be related to the latest model; more studies combining Ag titration and p-Syk measurements need to be done. In addition, Syk has an important role in regulating trafficking and processing of Ag internalized through the BCR (54). Active Syk might thus be required at the cSMAC to perform B cell–specific functions, not shared with its homolog in T cells, Zap70.

Syk kinase has a role in integrin activation downstream of the BCR and of chemokine receptors (32, 33). Our data indicate that the Syk activity level and localization determine vinculin recruitment at the B cell synapse. Syk promotes Bruton’s tyrosine kinase (Btk) recruitment to the plasma membrane (55). Btk associates with and transports PIPKI to the cell membrane to produce PIP2, the substrate of the Btk upstream activator PI3K. In this study, we show that vinculin recruitment parallels the PIP2 wave generated by PIPKιy early in synapse formation. Btk-mediated PIP2 production might thus support PI3K activity, but also assists vinculin localization to the synapse. In our model, above a certain threshold of tAg/BCR-promoted Syk activity, there is a local increase in Btk and PIPKιy-dependent PIP2 at the B cell–APC contact site; the PIP2 wave leads to vinculin recruitment and thus to pSMAC assembly and adhesion strength. This model explains our previous observations that below a tAg/BCR signaling threshold, B cells remain motile in response to chemokines and integrate BCR signals through the LFA-1–mediated migratory junction, the kinapse (7).

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure Legends

Supplemental Figure 1. IS assembly is necessary to arrest MD4 B cell motility. Primary MD4 B cells were allowed to settle on membranes containing GPI-ICAM-1 and coated with CXCL13, alone or with membrane-tethered (t)Ag or soluble (s)Ag. (A) Frequency of cell adhesion (estimated by IRM) and (B) cell polarization and motility (estimated by DIC microscopy) in the specified stimulation conditions. Data correspond to the merge of two experiments ±SEM. (C) Syk levels in primary B cells, untreated and treated with indicated stimuli, detected by western blot; see Methods for details. Tubulin was used as loading control. Data from a representative experiment (n = 3).

Supplemental Figure 2. Early recruitment of vinculin and F-actin to the B cell synapse. A20 B cells co-transfected with vinculin-GFP and Lifeact-RFP constructs in contact with ICAM-1/CXCL13 membranes and tAg were monitored for synapse formation by time-lapse confocal microscopy. (A) Time-lapse DIC, IRM, and fluorescence vinculin, F-actin and antigen images of a representative B cell are shown. (B) Representative profiles of total vinculin (top) and F-actin (bottom) fluorescence values at the B cell:membrane interface plane over time; dashed line indicates initiation of B cell interaction with the membrane (detected by IRM; considered time zero).

Supplemental Figure 3. Lentiviral infection of primary B cells for vinculin protein knockdown. (A) Endogenous vinculin and GFP reporter expression in B cells with indicated stimuli, detected by western blot 48 h after infection with lentiviral particles encoding non-silencing (scramble) and vinculin-silencing (clone V2LMM_56452) shRNA in the pGIPZ backbone; β-actin was used as loading control. Quantification of vinculin levels for each condition compared to non-silencing
(scramble)(bottom); see Methods for details. (B) B cells as in (A), with CpG stimuli, using lentiviral particles encoding non-silencing (scramble) and vinculin-silencing (1, clone NM_009502.3-3466s1c1; 2, clone NM_009502.3-1331s1c1; 3, clone NM_009502.3-3154s1c1; Mix, all three clones) shRNA in the pLKO.1 backbone; tubulin was used as loading control. Quantification of vinculin levels in each condition compared to non-silencing (scramble)(bottom). Data in (A) and (B) correspond to a representative experiment (n = 5).

**Supplemental Figure 4. Analysis of BAY treatment in primary B cells.** (A) p-ERK1/2 levels in primary B cells, untreated and treated with indicated BAY doses and stimulated with sAg. Quantification of p-ERK1/2 (bottom) for each condition compared to basal control (no BAY treatment, no sAg stimulation); see Methods for details. Tubulin was used as loading control. Data from a representative experiment. (B) Migration frequency of untreated and BAY-treated primary B cells in contact with ICAM-1/CXCL13 membranes (left). Mean velocity of the motile B cells at the left; each dot represents a single cell (right). Data correspond to the merge of two experiments. (C) Total vinculin (left) and F-actin (right) fluorescence values at the contact plane (in arbitrary units, AU) of untreated and BAY-treated (doses specified) B cells settled on ICAM-1/CXCL13 membranes with tAg; each dot represents a single cell. Data correspond to the merge of two experiments. (D) DIC and fluorescence vinculin (FITC), F-actin (AlexaFluor647) and antigen (AlexaFluor555) images of a representative BAY-treated (0.1 µM) B cell forming a synapse on ICAM-1/CXCL13 membranes with tAg. Statistical analysis, two-tailed unpaired Student t-test was applied (Prism 4.0); *, p<0.05; ***, p<0.0001.
Supplemental Video Legends

Supplemental Video 1. CXCL13-mediated B cell migration on ICAM-1/CXCL13 membranes. Primary B cells migrating on ICAM-1/CXCL13 membranes. DIC and IRM images over time (15 min; 7 frames/sec).

Supplemental Video 2. sAg/BCR stimulation does not arrest CXCL13-mediated B cell motility. Behavior of primary B cells settled on ICAM-1/CXCL13 membranes with sAg. DIC and IRM images over time (15 min; 7 frames/sec); arrows indicate B cell motility.

Supplemental Video 3. tAg/BCR stimulation abolishes B cell motility. Behavior of primary B cells settled on ICAM-1/CXCL13 membranes with tAg, showing an established synapse. DIC and IRM images over time (15 min; 7 frames/sec).

Supplemental Video 4. Vinculin and F-actin recruitment during B cell synapse formation. A20 B cells co-transfected with vinculin-GFP and Lifeact-RFP constructs, settled on ICAM-1/CXCL13 membranes with fluorescent tAg, were tracked for synapse formation. DIC, IRM and fluorescence images of a representative A20 B cell for vinculin (green), F-actin (red) and antigen (white) over time (18 min; 5 frames/sec).

Supplemental Video 5. Early PIPKIγ recruitment wave and distribution at the B cell synapse. A20 B cells transfected with PIPKIγ-GFP construct, settled on ICAM-1/CXCL13 membranes with fluorescent tAg were tracked for synapse formation. DIC, IRM and fluorescence images of a representative A20 B cell for PIPKIγ (color-coded) and antigen (red) over time (8 min; 5 frames/sec).

Supplemental Video 6. PIP2 production wave and distribution at the B cell synapse. A20 B cells transfected with the PIP2 probe PLCδ-PH-GFP construct, settled on ICAM-1/CXCL13 membranes with fluorescent tAg, were tracked for
synapse formation. DIC, IRM and fluorescence images of a representative A20 B cell for PIP2 (color-coded) and antigen (red) over time (9 min; 5 frames/sec).

**Supplemental Video 7. Dynamics of BAY-untreated B cells with synapse established.** Behavior of primary B cells settled on ICAM-1/CXCL13 membranes with fluorescent tAg. DIC and overlaid IRM/fluorescent tAg (AlexaFluor555) images over time (10 min; 7 frames/sec); arrowhead indicates dynamics of a representative B cell with synapse formed (red antigen cluster, cSMAC) and CXCL13-induced membrane ruffles (detected by IRM and DIC).

**Supplemental Video 8. Dynamics of BAY-treated B cells with tAg.** Behavior of BAY-treated primary B cells settled on ICAM-1/CXCL13 membranes with fluorescent tAg. DIC and overlaid IRM/fluorescent tAg (AlexaFluor555) images over time (10 min; 7 frames/sec); arrowheads show dynamics of representative B cells migrating in response to CXCL13 and bearing the antigen cluster (red) at the uropod.

**Supplemental Video 9. NM-II inhibition promotes vinculin loss from the synapse.** A20 B cells co-transfected with vinculin-GFP and Lifeact-RFP constructs, settled on ICAM-1/CXCL13 membranes with fluorescent tAg, were allowed to form the synapse, treated with blebbistatin and tracked for vinculin and F-actin molecular dynamics. DIC, IRM and fluorescence images of a representative blebbistatin-treated A20 B cell for vinculin (green), F-actin (red) and antigen (white) over time (20 min; 5 frames/sec); time frames for blebbistatin addition and the 10-min time point are shown.
Supplemental Figure 1. Saez de Guinoa et al.
### A

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**Western Blot Analysis**

- **Vinculin**: Shown with variations in conditions.
- **β-actin** and **GFP** as loading controls.

**Ratio for Vinculin**

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**Western Blot Analysis**

- **Vinculin** and **Tubulin** as loading controls.

**Ratio for Vinculin**

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**Supplemental Figure 3. Saez de Guinoa et al.**