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Article

B Cell Receptor-Mediated Antigen Gathering Requires Ubiquitin Ligase Cbl and Adaptors Grb2 and Dok-3 to Recruit Dynein to the Signaling Microcluster

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SUMMARY

The B cell receptor (BCR) mediates B cell antigen gathering and acquisition for presentation to T cells. Although the amount of antigen presentation to T cells determines the extent of B cell activation, the molecular mechanisms underlying antigen gathering remain unexplored. Here, through a combination of high-resolution imaging, genetics and quantitative mass spectrometry, we demonstrate that adaptors Grb2 and Dok-3, and ubiquitin ligase Cbl in signaling BCR microclusters mediate association with the microtubule motor dynein. Furthermore, we visualize the localization and movement of these microclusters on the underlying microtubule network. Importantly, disruption of this network or diminished dynein recruitment in Grb2-, Dok-3-, or Cbl-deficient B cells, does not influence microcluster formation or actin-dependent spreading, but abrogates directed movement of microclusters and antigen accumulation. Thus we identify a surprising but pivotal role for dynein and the microtubule network alongside Grb2, Dok-3, and Cbl in antigen gathering during B cell activation.

INTRODUCTION

On sensing cognate antigen, B cells proliferate and differentiate to form antibody-secreting plasma cells and long-lasting memory cells (Rajewsky, 1996). This activation process requires exquisite regulation to finely balance the need for rapid protective responses to prevent infection while simultaneously prohibiting spurious uncontrolled activation associated with autoimmune disease.

Substantial recent progress using advanced imaging techniques has uncovered molecular events during early B cell activation. Regardless of the state of the B cell receptor (BCR) in the resting B cell membrane (Tolar et al., 2005; Yang and Reth, 2010), the actin cytoskeleton restricts BCR diffusion and disruption of this network is sufficient to initiate intracellular signaling (Treanor et al., 2010). Following recognition of antigen, discrete BCR microclusters containing small numbers of antigen molecules form throughout the contact interface (Depoil et al., 2008). The formation of these microclusters does not require signaling through the BCR (Depoil et al., 2008), and it has been suggested clustering may be influenced by conformational changes in the BCR constant region (Tolar et al., 2009). BCR microclusters recruit constituents of the B cell signalosome (reviewed in Kurosaki, 1999) such as Lyn, Syk, Vav, and PLCγ2 (Weber et al., 2008). The initiation of BCR signaling stimulates rapid actin-dependent B cell spreading, increasing the amount of antigen contacted and the number of microclusters formed (Fleire et al., 2006; Weber et al., 2008). The extent of spreading determines the amount of antigen gathered at the central cluster of the mature immunological synapse (IS) (Fleire et al., 2006). This structure, initially observed in $CD4^+$ T cells, is recognized as a common feature of lymphocyte activation (Monks et al., 1998). In B cells, the central cluster is particularly important because gathered antigen is acquired through the BCR (Batista et al., 2001) and trafficked to MHC-containing lysosomes in a mechanism thought to involve myosin II (Vascotto et al., 2007). Processed antigen in complex with MHC is then presented to $CD4^+$ helper T cells for maximal B cell activation (Lanzavecchia, 1985).

Although we are gaining some grasp of the early molecular events of B cell activation and in particular the importance of actin reorganizations, the antigen-gathering process and concomitant contraction remain unexplored. Our current understanding of the mechanism by which BCR microclusters are gathered into a central cluster is based on observations in T cells. Because actin polymerization occurs at the leading edge, TCR microclusters associated with actin filaments are thought to move passively to the central cluster via centripetal retrograde actin flow (Kaizuka et al., 2007). Indeed, signaling microclusters comigrate with retrograde actin flow and this

migration is arrested on disruption of the actin cytoskeleton (Varma et al., 2006). Furthermore, myosin IIA may participate in the active transport of TCR microclusters along actin filaments (Ilani et al., 2009). Although these observations are potentially informative, the process of antigen gathering in T and B cells differs significantly and thus may be mediated by different underlying mechanisms. Whereas in T cells sustained signaling occurs in newly generated peripheral microclusters and antigen gathering results in signal attenuation (Varma et al., 2006), in B cells microclusters are rapidly gathered on completion of spreading during contraction (Fleire et al., 2006).

Here, we have identified a role for the motor protein dynein in mediating the transport of BCR microclusters along the microtubule network for antigen gathering. The recruitment of dynein for BCR microcluster movement requires the adaptor protein Grb2 and the associated mediators Dok-3 and Cbl. Because antigen gathering is required for subsequent acquisition and presentation to T cells, we have established a critical role for dynein and the microtubule network during B cell activation.

RESULTS

Grb2, Dok-3, and Cbl Participate in Movement of Microclusters to Gather Antigen

To identify molecular players important for the accumulation of antigen in the central cluster, we examined a large pre-existing panel of DT40 chicken B cells lacking selected molecules (Shinohara and Kurosaki, 2006) for defects in antigen gathering.

B cells were settled on planar lipid bilayers containing laterally mobile fluorescent-labeled surrogate antigen (M1 monoclonal anti-chicken BCR), and subsequently antigen distribution and contact with the bilayer were visualized with confocal and interference reflection microscopy (IRM), respectively. In wild-type (WT) B cells, antigen-containing microclusters formed during spreading are gathered together and accumulate in a central cluster concomitant with B cell contraction (Figure 1A). In contrast to Lyn, Syk, PLCγ2, and Vav that mediate B cell spreading (Weber et al., 2008), we identified three mediators required for antigen gathering: the small molecular adaptor protein growth factor receptor-bound protein 2 (Grb2) (Lowenstein et al., 1992); the third member of the Dok family of adaptor proteins Dok-3 (Lemay et al., 2000); and an adaptor of the ubiquitin ligation machinery Casitas B cell lymphoma (Cbl) (Tezuka et al., 1996) (Figures 1B–1D). Although *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, or *Cbl*$^{-/-}$B cells are able to spread as WT B cells (Figure 1E) they exhibit significant impairment in contraction and antigen accumulation (Figures 1E and 1F; Movie S1 available online). Importantly, reconstitution of these cells with a WT version of the mediator restores antigen gathering and also contraction (see below). It is worth noting that similar defects were not observed in other cells in the panel that lack mediators including PI3K, BCAP, and Bam32 (data not shown).

We moved on to examine whether the inability of *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, or *Cbl*$^{-/-}$ B cells to gather antigen was a result of a defect in the generation of antigen microclusters or in their subsequent movement to the center. To improve the effective resolution, we used total internal reflection fluorescence microscopy (TIRFM) to visualize antigen microclusters in *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, or *Cbl*$^{-/-}$ B cells settled on antigen-containing lipid bilayers (Figure 1G). In line with the initial screen (Figures 1B–1E), these cells were not impaired in their spreading response and in addition were able to form antigen-containing microclusters to the same extent as WT cells (Figure 1G, upper panels, and Figure S1). However, 15 minutes after stimulation, these microclusters remained in the periphery in the absence of Grb2, Dok-3, or Cbl (Figure 1G, lower panels). Furthermore, unlike in WT cells, microclusters tended not to undergo directed net movement toward the center of contact (Figure 1H).

These observations suggest that, though dispensable for spreading and formation of antigen-containing microclusters, Grb2, Dok-3, and Cbl participate in B cell contraction and the efficient movement of microclusters for antigen gathering.

Dok-3 and Cbl Recruitment to Antigen-Containing Microclusters Is Dependent on Grb2

A role for Grb2, Dok-3, and Cbl in gathering BCR microclusters is particularly interesting given their role in the downregulation of BCR signaling (Stork et al., 2007; Yasuda et al., 2000). To visualize their distribution during activation, we settled *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, or *Cbl*$^{-/-}$ B cells reconstituted with endogenous levels of fluorescent-labeled protein on antigen-loaded bilayers. We observed the three mediators localized in antigen microclusters during B cell spreading (Figures 2A–2C; Figure 2D, filled circles; Movie S2); however, consistent with its N-terminal PH domain, a proportion of antigen-independent Dok-3 clusters and Cbl clusters were also visible at the membrane (Figure 2D, empty circles). Interestingly, after cell contraction, whereas both Grb2 and Cbl were predominantly present with accumulated antigen, Dok-3 was absent from this region and presumably dissociates from antigen-containing microclusters as they move toward the center (Figure 2A–2C, lower panels). In view of the early recruitment of Grb2, Dok-3 and Cbl to the microcluster, it seems likely that these mediators may participate as part of a functional complex. This suggestion is in line with previously characterized interactions for Grb2 in stimulated B cells (Panchamoorthy et al., 1996; Stork et al., 2007) and indeed Cbl was copurified from activated B cells expressing Grb2-GFP or Dok-3-GFP, but not from *Grb2*$^{-/-}$ or *Dok3*$^{-/-}$ B cells, with anti-GFP for capture (Figure 2E).

To investigate the molecular requirements for recruitment of Grb2, Dok-3, and Cbl to the antigen microcluster, we visualized the distribution of fluorescent-labeled versions of these mediators in DT40 B cells lacking various signaling molecules after settling them on antigen-containing lipid bilayers. We observed that Dok-3 and Cbl recruitment to the antigen microcluster is significantly impaired in *Grb2*$^{-/-}$ B cells (Figures 3A and 3B). While Dok-3 forms clusters in the membrane in Grb2-deficient B cells, colocalization of these clusters with antigen microclusters is reduced (Figure 3A). Similarly, the limited number of diffuse clusters formed in *Grb2*$^{-/-}$ B cells expressing Cbl-YFP do not colocalize with antigen microclusters (Figure 3B). Furthermore, overexpression of either Dok3-GFP or Cbl-YFP is not sufficient for restoring antigen gathering in *Grb2*$^{-/-}$ cells (data not shown). Importantly, the recruitment of Grb2-GFP to the microcluster was abrogated in *Lyn*$^{-/-}$ B cells, although these cells form a limited number of antigen microclusters in response to stimulation (Figure 3C) (Weber et al., 2008). This indicates that BCR-mediated signaling is necessary for Grb2 localization to

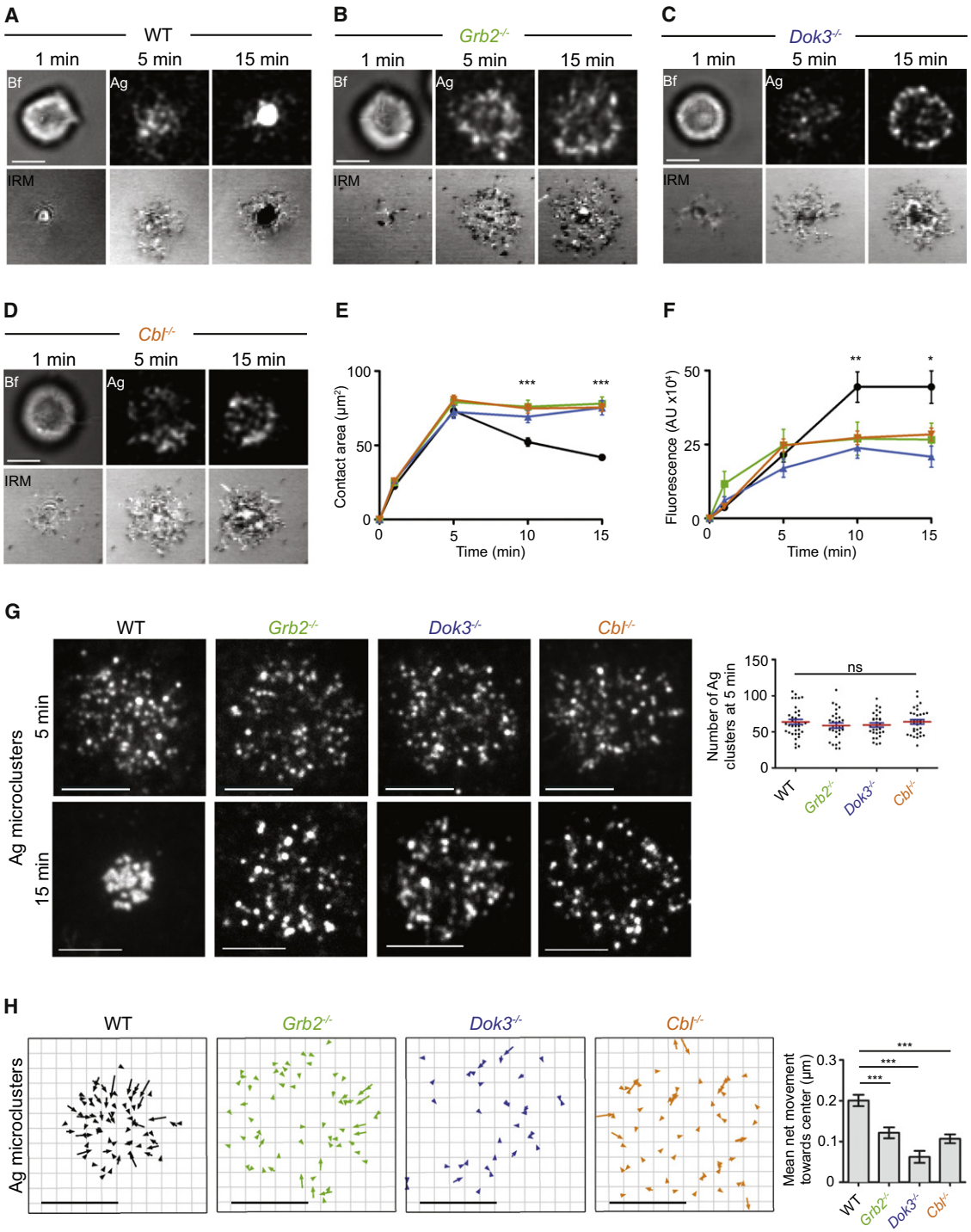

Figure 1. Grb2, Dok-3, and Cbl Participate in Movement of Microclusters to Gather Antigen

(A–H) DT40 B cells were settled on lipid bilayers containing anti-IgM (antigen, Ag).

(A–D) Brightfield (Bf) and confocal (upper), and IRM (lower) images of (A) WT (black), (B) *Grb2*$^{-/-}$ (green), (C) *Dok3*$^{-/-}$ (blue), and (D) *Cbl*$^{-/-}$ (orange) B cells.

(E and F) Quantification of (E) contact area and (F) antigen gathered from (A)–(D). In each case at least 25 cells acquired from three independent experiments were examined.

(G) TIRFM images of WT, *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, and *Cbl*$^{-/-}$ B cells and mean number of Ag microclusters (±SEM) in each cell was counted.

(H) Ag microcluster movement between 5–6 min as (left panel) vectors and (right panel) mean net movement toward cell center (± SEM). Data from at least 350 tracks in 10 cells over two independent experiments. The scale bar represents 5 μm. Student's t test: ***p < 0.0001; **p < 0.005; *p < 0.02. See also Figure S1 and Movie S1.

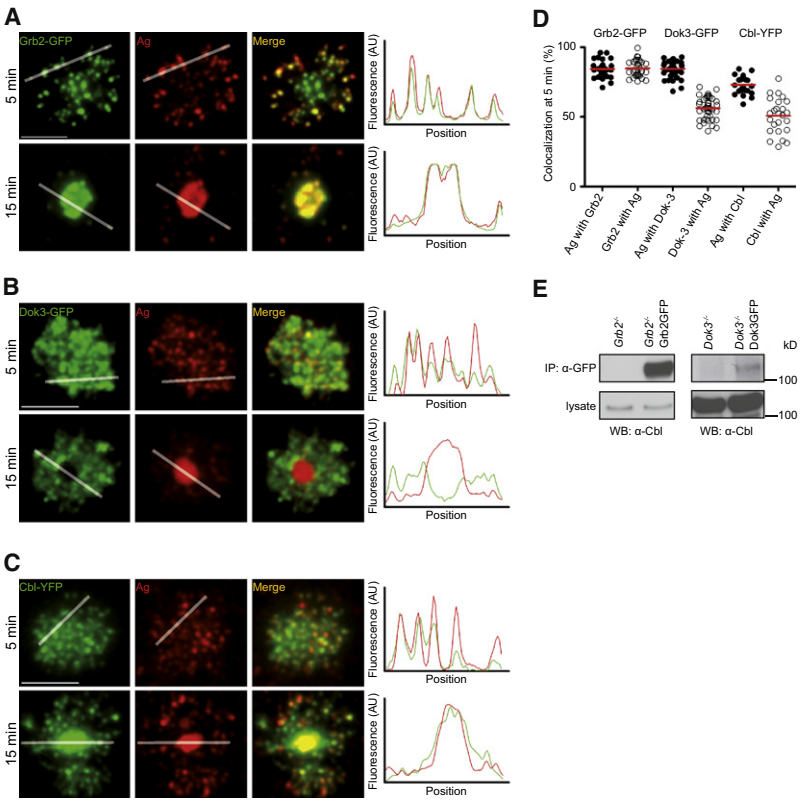

Figure 2. Grb2, Dok-3, and Cbl Act in a Complex that Is Recruited to Antigen-Containing BCR Microclusters

(A–D) DT40 B cells were settled on lipid bilayers containing anti-IgM (Ag).

(A–C) TIRFM images and relative fluorescence intensity along white line in (A) *Grb2*$^{-/-}$ with Grb2-GFP, (B) *Dok3*$^{-/-}$ with Dok3-GFP, and (C) *Cbl*$^{-/-}$ with Cbl-YFP are shown. Scale bars represent 5 μm.

(D) Colocalization of Grb2-GFP, Dok3-GFP, and Cbl-YFP with Ag microclusters (filled circles) and vice versa (empty circles). Each point is one cell, pooled from at least three independent experiments (mean in red).

(E and F) Western blots to detect Cbl in (upper) immunoprecipitates (IP) with anti-GFP and (lower) stimulated DT40 B cell lysates.

(E) *Grb2*$^{-/-}$ with or without Grb2-GFP (left) and *Dok3*$^{-/-}$ with or without Dok3-GFP (right). See also Movie S2.

the antigen-containing microcluster. In addition, the recruitment of Grb2 to the microcluster was abrogated upon expression of an inactivating mutation in the central SH2 domain of Grb2 (R86K; Figure 3D). Furthermore, although Grb2R86K did not impair the formation of antigen microclusters or the spreading response, it was unable to restore antigen gathering or contraction in *Grb2*$^{-/-}$ B cells (Figures 3E–3G). Interestingly, recruitment of Grb2-GFP to signaling microclusters was altered in *Dok3*$^{-/-}$ B cells, suggesting that multiple cooperative interactions are important within the microcluster (Movie S3). However, we observed that *Dok3*$^{-/-}$ and *Cbl*$^{-/-}$ B cells were not altered in their recruitment of Cbl or Dok-3 and Grb2 to antigen microclusters, respectively (Figures S2A–S2C).

Taken together, these observations suggest that early B cell signaling is mandatory for Grb2 recruitment to the antigen microcluster, and such recruitment is required for the localization of the associated mediators Dok-3 and Cbl at the microcluster.

Signaling BCR Microclusters Recruit the Microtubule Motor Dynein and Interact with Tubulin

To understand why the absence of Grb2, Dok-3, and Cbl at the BCR microcluster renders cells unable to gather antigen, we have taken advantage of a recent approach to elucidate the Syk interactome by using stable isotype labeling by amino acids in cell culture (SILAC) followed by high-end mass spectrometry (T.O., H.U., J.W., unpublished data). Syk can be used as a molecular marker of signaling BCR microclusters because it directly binds to phosphorylated ITAMs in the BCR, is rapidly recruited to BCR microclusters after stimulation, and has been shown to be ubiquitinated by Cbl (Kitaura et al., 2007). In this approach, *Syk*$^{-/-}$ DT40 B cells were reconstituted with a STrEP-One tagged version of Syk and cultured in "heavy" medium, whereas WT DT40 B cells cultured in "light" medium served as a control (see Experimental Procedures). After stimulation, the cell lysates were passed over a Strep affinity column and binding fractions were pooled in a 1:1 ratio and subjected to trypsin digestion followed by liquid-chromatography-coupled tandem mass spectroscopy (MS-MS). Using this differential labeling setup, one can quantify the enrichment of a particular peptide relative to the WT background and thus identify specific binding partners for Syk (Neumann et al., 2009). Importantly, peptides derived from Syk alongside known specific interaction partners such as the BCR itself were identified in the digested MS-MS sample (Figure 4A), whereas unrelated proteins such as the ribosomal protein L10 were not enriched above background (Figure S3A).

Our attention was drawn to the enrichment of a number of peptides derived from several tubulins in the Syk interactome, as this establishes a potential link between BCR microclusters and the microtubule network (Figure 4A; Table S1). In line with this, Syk has been shown to mediate α-tubulin phosphorylation after BCR stimulation (Peters et al., 1996). Interestingly, we also observed a 13.8- and 1.8-fold enrichment of peptides corresponding to the heavy chain of cytoplasmic dynein (DYNC1H1) and the dynactin subunit 2 (DCTN2; dynamitin), respectively (Figure 4A; Table S1). These interactions were of particular significance given that dynein is a minus-end-directed motor protein directing the transport of cargo along microtubules toward the microtubule-organizing center (MTOC). In line with the SILAC MS-MS data, we detected the dynein intermediate chain of 74 kDa (DYNC1I1) in Syk immunoprecipitates from activated B cells (Figure 4B).

In support of this, we also observed that glutathione beads coated with Grb2-GST captured dynein from lysates of DT40 B cells stably expressing GFP-tagged DYNC1I1 (Figure S3B), and the C-terminal SH3 domain of Grb2 is necessary

for this capture (Figure S3B). Furthermore, as DYNC1I1 immunoprecipitated with Dok-3, dynein appears a bona fide binding partner for signaling microclusters (Figure S3C). In addition, the $p150^{Glued}$ subunit of dynactin (DCTN1) immunoprecipitated with both Grb2-GFP and Dok3-GFP, suggesting that dynein is functional at the microcluster (Figures S3D and S3E). Somewhat surprisingly the Grb2 interactome elucidated by SILAC MS-MS did not detect specific enrichment of dynein, dynactin or tubulins (data not shown; Neumann et al., 2009). In line with this we did not observe DYNC1I1 in immunoprecipitates of Grb2 (data not shown). Thus, although the dynein-dynactin complex interacts with signaling microclusters, this does not seem to be by direct physical association with Grb2, Dok-3, and Cbl.

Because dynein is most commonly associated with the transport of intracellular cargo along microtubules, the association with signalosome components raises the intriguing possibility that dynein and dynactin participate in microcluster movement during antigen gathering. To investigate this, we transduced primary mouse B cells with a retroviral vector coding for DYNC1I1-GFP and settled them onto planar lipid bilayers containing fluorescent-labeled antigen (anti-mouse BCR κ chain) and ICAM-1. In line with the SILAC MS-MS and biochemical approaches, we observed that DYNC1I1-GFP was indeed colocalized with antigen microclusters (Figure 4C). Furthermore, DYNC1I1-GFP migrates with antigen microclusters toward the center of the cell (Figure 4D; Movie S4). Together, these three complementary approaches identify a potential interaction between the antigen-containing microcluster and the dynein complex.

Antigen-Containing Microclusters Move Along Microtubules after B Cell Spreading

The localization of dynein to the signaling microcluster suggests a role for the microtubule network in organizing microcluster movement during antigen gathering. To explore this possibility, we settled primary mouse B cells expressing tubulin-RFP on planar lipid bilayers containing fluorescent-labeled antigen and unlabelled ICAM-1. Using TIRFM, we often observed antigen-containing microclusters on and moving along microtubules over time (Figure 5A). However, this movement was not continuous because the microtubule network was highly dynamic and frequently disappeared from the focal plane (Figure 5A; Movie S5). Interestingly, single particles of BCR were immobilized in tubulin-rich regions after settling on glass coverslips coated with fibronectin in the presence of surrogate antigen (Figure S4A; Movie S6). This is in line with an interaction between the microtubule network and the microcluster and suggests a potential role for tubulin during the formation of BCR microclusters.

To visualize the gross morphology of the microtubule network at high resolution, we settled murine B cells expressing tubulin-cherry on a glass coverslip coated with surrogate antigen and allowed them to reach maximum spread prior to imaging using structured illumination microscopy (SIM) (Gustafsson et al., 2008). These imaged B cells also expressed a marker of filamentous actin (LifeAct-GFP) (Riedl et al., 2008) and thus showed the distribution of the microtubule network relative to the actin cytoskeleton. We observed two distinct areas at the contact surface: a central region occupied by the microtubule network and a surrounding tubulin-scarce zone containing filamentous actin. This distribution is consistent with actin polymerization at the leading edge driving B cell spreading (Fleire et al., 2006) (Figure 5B). After spreading, tubulin is often polarized toward the antigen-coated surface, probably reflecting the localization of the repositioned MTOC after stimulation. However, an extended slice y-projection image revealed that microtubules exhibit a wave-like behavior under the B cell membrane and periodically come in close proximity to the contact interface (Figure 5B, black arrows), providing a potential explanation for the discontinuous nature of microcluster movement on the microtubule network detected with TIRFM (Figure 5A). A similar polarized distribution of dynein was also observed in DT40 B cells expressing DYNC1I1-GFP (Figure S4B).

The distribution of the microtubule network is consistent with a role in antigen gathering, and indeed antigen-containing microclusters colocalize and move with tubulin after B cell spreading.

Dynein and the Microtubule Network Participate in Antigen Gathering

To investigate the role of dynein and the microtubule network in B cells, we examined the extent of antigen gathering after disruption of the microtubule network as well as inhibition of dynein expression and activity.

Pretreatment of primary murine B cells with the microtubule-stabilizing drug taxol prior to settling on lipid bilayers containing surrogate antigen and ICAM-1 significantly reduced gathering of antigen-containing microclusters and accumulation of antigen (Figure 5C). Similarly, treatment with the microtubule inhibitor nocodazole prevents movement of microclusters to the central region (Figure S4C). However, although microtubule-modifying agents prevent B cell contraction, they do not appear to impair either the formation of antigen microclusters (Figure 5D) or the extent of actin-dependent B cell spreading (Figures 5C and 5D; Figure S4C).

To investigate the role of dynein in antigen gathering, we used two alternative approaches. The first involved overexpression of the dynamitin subunit (p50) of the dynactin complex that has a dominant-negative effect on dynein function. The second approach involved silencing dynein expression by transfection of murine A20 B cells with shRNA targeting DYNC1H1. This construct includes an IRES-GFP that allows B cells to be sorted according to shRNA expression 2 days prior to assessing the extent of dynein knockdown by western blotting (Figures 6B and 6C). A20 B cells either overexpressing dynamitin-GFP (Figure 6A) or expressing DYNC1H1 shRNA (Figure 6C and Figure S5) were settled on lipid bilayers containing fluorescent-labeled antigen and movement of microclusters was monitored (Figure 6D). We observed that the net movement of microclusters toward the center was severely impaired after disruption of dynein expression or function (Figure 6E and Movie S7), implying a function for dynein in mediating antigen gathering.

Thus, treatment with microtubule inhibitors and disruption of dynein together provides evidence for a role for dynein and the microtubule network in antigen gathering, and intriguingly replicates the phenotypes observed in *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, or *Cbl*$^{-/-}$ B cells (Figure 1).

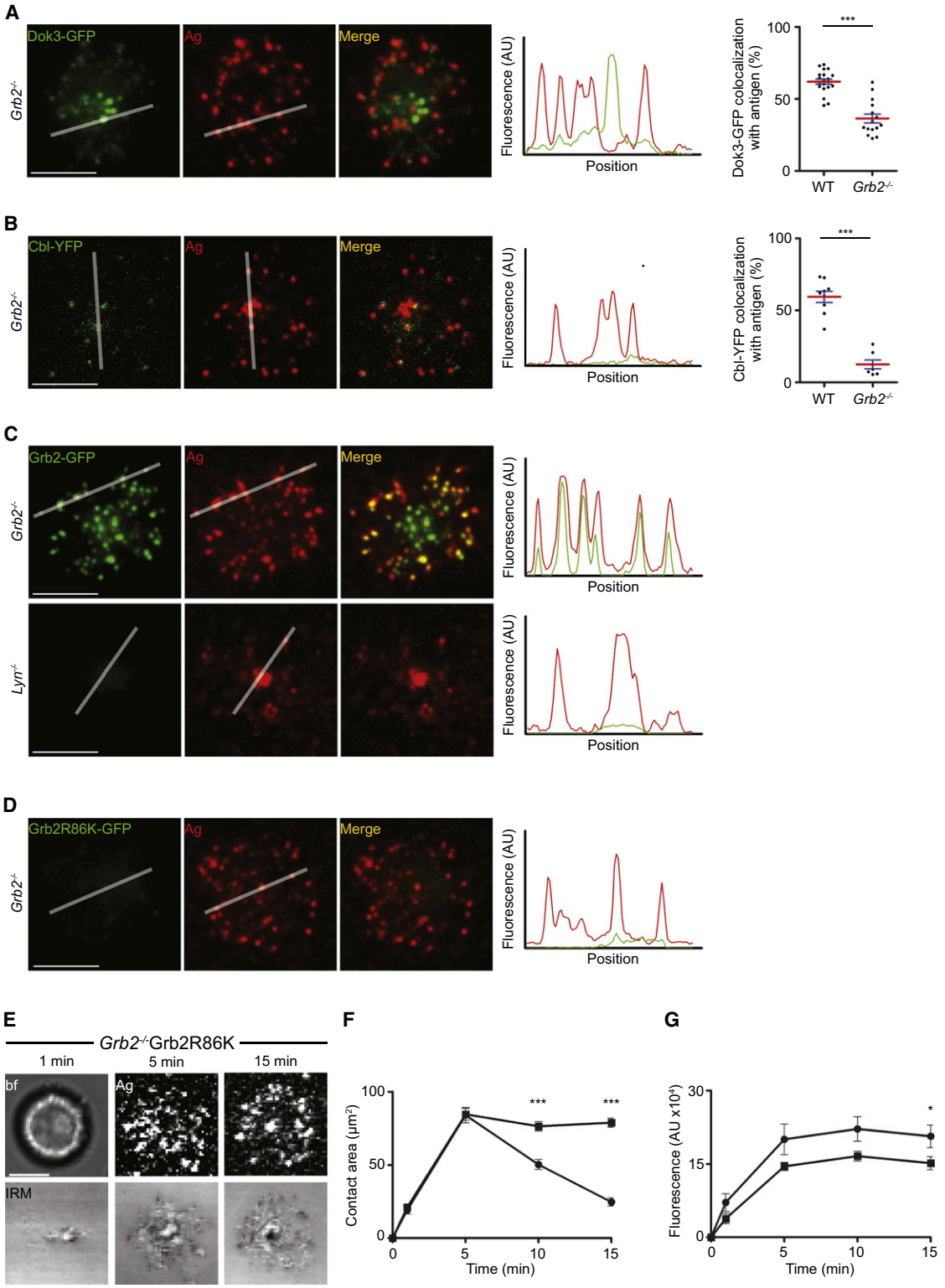

Figure 3. Dok-3 and Cbl Recruitment to Antigen Microclusters Is Dependent on Grb2 and Requires BCR Signaling

(A–D) DT40 *Grb2*$^{-/-}$ B cells were settled on lipid bilayers containing anti-IgM (Ag). TIRFM images and relative fluorescence intensity along white line in cells expressing (A) Dok3-GFP, (B) Cbl-YFP, (C) Grb2-GFP and (D) Grb2R86K-GFP are shown.

(A and B) Colocalization of Dok3-GFP and Cbl-YFP with Ag between 4 and 7 min is shown on the right panels. Each point is one cell, pooled from at least two independent experiments (mean in red ± SEM).

(C) As shown on the lower panels, DT40 *Lyn*$^{-/-}$ B cells expressing Grb2-GFP were settled on lipid bilayers containing Ag.

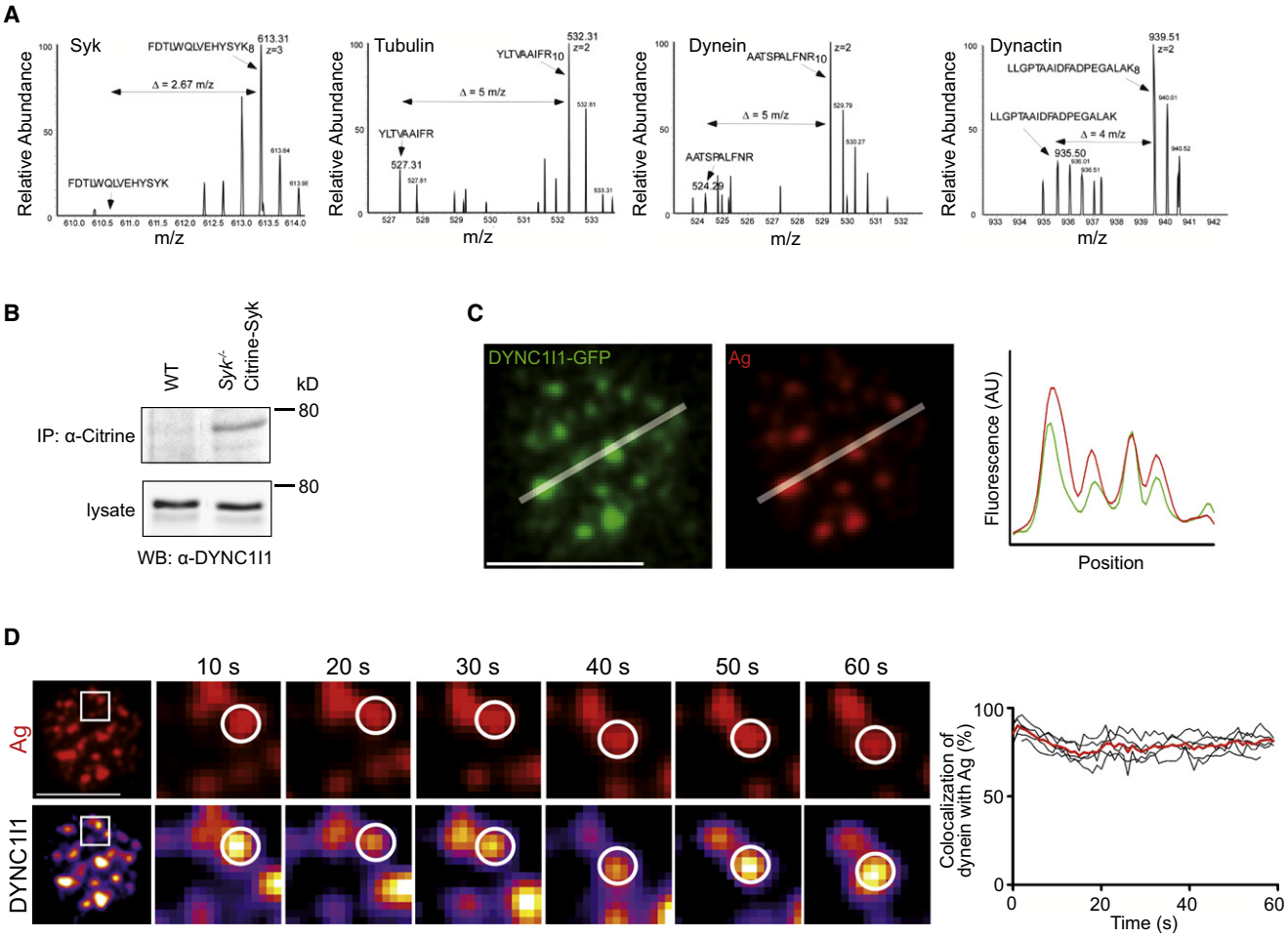

Figure 4. Signaling BCR Microclusters Interact with Tubulin and Recruit the Microtubule Motor Dynein

(A) Quantitative MS-MS for enriched peptides derived from Syk, tubulin β2, DYNC1H1 (Dynein), and Dctn2 (Dynactin) with "heavy" and "light" mass peaks identified.

(B) Western blots for DYNC1I1 in (upper) immunoprecipitates (IP) with anti-Citrine and (lower) stimulated lysates from WT or *Syk*$^{-/-}$ DT40 B cells expressing Citrine-Syk.

(C and D) Primary murine B cells expressing DYNC1I1-GFP were settled on lipid bilayers containing anti-κ (Ag) and ICAM-1.

(C) TIRFM images and relative fluorescence intensity along white line at 4 min.

(D) As shown on the left, TIRFM image with Ag (red) and DYNC1I1-GFP (pseudocolor according to intensity) with time-lapse magnifications from white box highlighting Ag-DYNC1I1-GFP colocalization (white circle). As shown on the right, Ag colocalization with DYNC1I1-GFP over time (single cells, black; mean, red). The scale bar represents 5 μm. See also Figure S3 and Movie S4.

Dynein Localization to the BCR Microcluster Is Required for Antigen Gathering

The inhibition studies suggest that while the microtubule network does not play a key role during the spreading response early in B cell activation, it participates in subsequent antigen gathering.

Thus far, our observations suggest that the microtubule network makes up tracks along which microclusters move for antigen gathering, although it was not clear why the absence of Grb2, Dok-3, and Cbl impairs antigen microcluster movement (Figure 1; Figure 5). Because this could be due to an alteration of the underlying microtubule network, we settled *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, or *Cbl*$^{-/-}$DT40 B cells stably expressing tubulin-RFP on planar lipid bilayers containing antigen. Surprisingly TIRFM clearly revealed that the gross organization of the microtubule network was not disrupted and antigen microclusters localized with tubulin-rich regions (Figure 7A; Movie S8).

Because antigen gathering is severely impaired on disruption of dynein (Figure 6), we examined the distribution of dynein in an effort to uncover the molecular explanation for the observed defective phenotype in *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, or *Cbl*$^{-/-}$ DT40 B cells

(E–G) DT40 *Grb2*$^{-/-}$ B cells expressing Grb2R86K were settled on lipid bilayers containing Ag.

(E) Bf and confocal (upper) and IRM (lower) images.

(F and G) Quantification of (F) contact area and (G) antigen gathered from E with WT cells (circles), *Grb2*$^{-/-}$ expressing Grb2R86K (squares). Data are from at least 20 cells and three independent experiments. Student's t test: ***$p < 0.0001$; *$p < 0.02$. The scale bar represents 5 μm. See also Figure S2 and Movie S3.

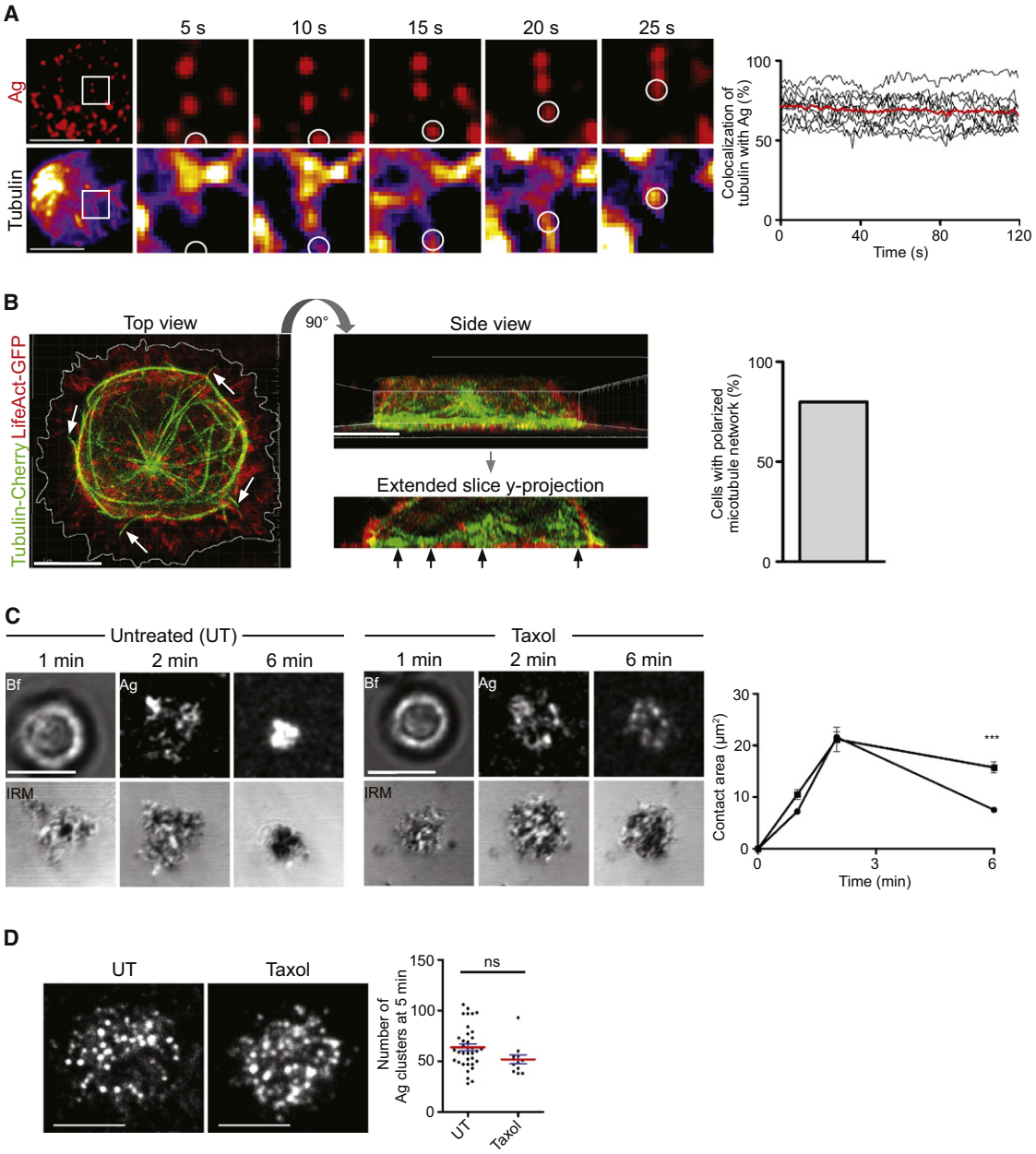

Figure 5. Antigen-Containing Signaling Microclusters Are Moved along the Microtubule Network after B Cell Spreading

(A) Primary murine B cells expressing α-tubulin-RFP (pseudocolor according to intensity) were settled on lipid bilayers containing anti-κ (Ag, red) and ICAM-1. As shown on the left, TIRFM image at 4 min, with time-lapse magnifications from white box highlighting Ag-α-tubulin-RFP co-localization (white circle). As shown on the right, Ag co-localization with α-tubulin-RFP over time (single cells, black; mean, red).

(B) SIM projection images of murine A20 B cells expressing tubulin-cherry and LifeAct-GFP settled on glass coated with Ag, with microtubule protrusions (white arrows) and membrane-proximal protrusions (black arrows). Shown on the right is the frequency of microtubule network polarization toward the interface from 21 cells.

(C and D) Primary murine B cells with or without pretreatment with 59 μM taxol, were settled on lipid bilayers containing anti-κ (Ag) and ICAM-1.

(C) Shown on the left are Bf and confocal (upper) and IRM (lower) images. Shown on the right, quantification of contact area from IRM images. Data are from at least 25 cells over two independent experiments.

(D) TIRFM (left) images at 5 min and (right) quantification of Ag microclusters with each point as a single cell (mean, red; ± SEM, blue). Student's t test: ***$p < 0.0001$; ns, not significant. The scale bar represents 5 μm. See also Figure S4 and Movie S5 and Movie S6.

(Figures 1B–1D). We settled these cells stably expressing matched amounts of DYNC1I1-GFP on planar lipid bilayers containing surrogate antigen to visualize the distribution of dynein relative to antigen microclusters (Figure 7B and Figure S6A). Images were acquired every second for a minute after cells had reached maximum spread. As shown in Figure 7B, dynein was

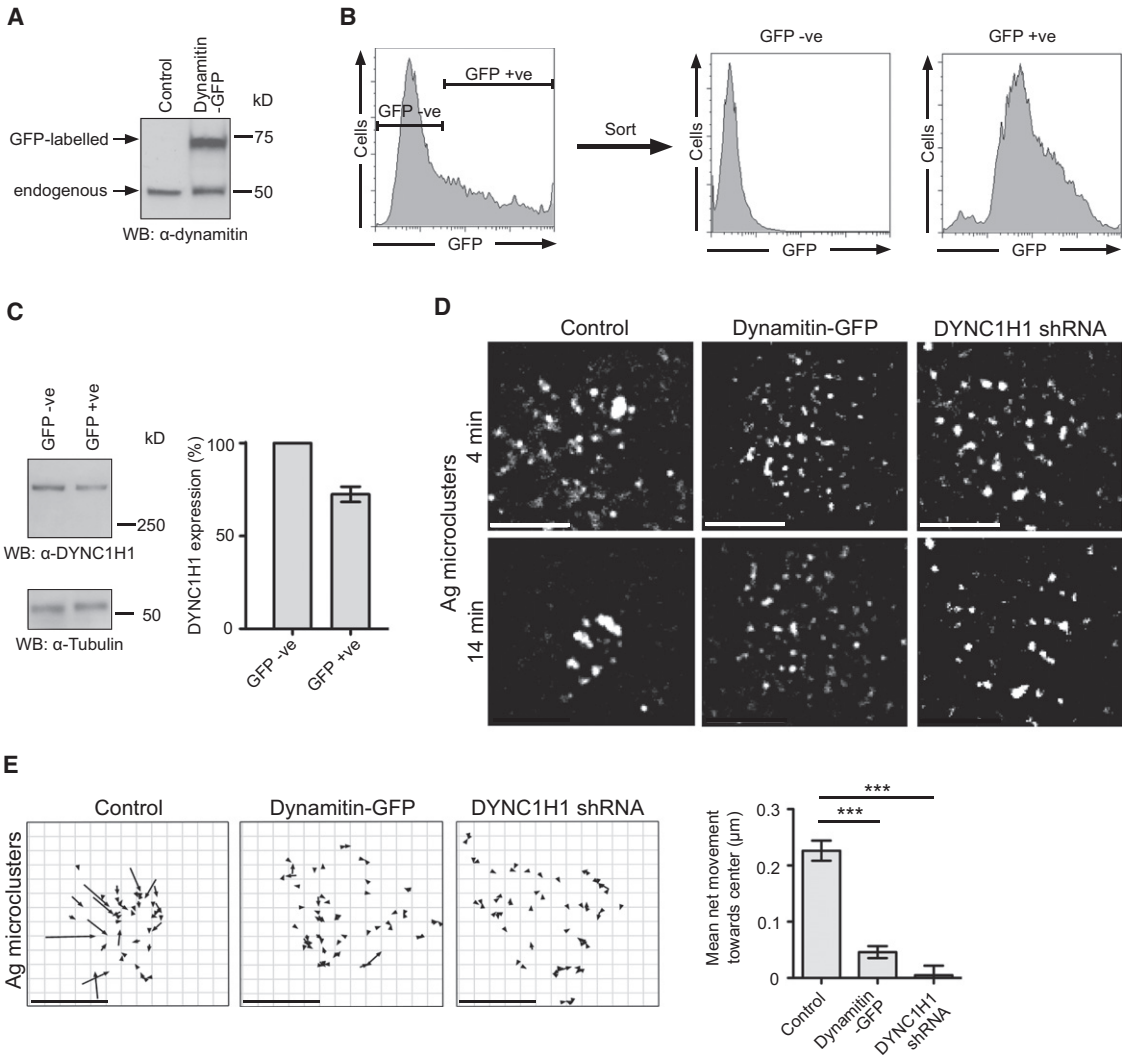

Figure 6. Dynein Is Involved in Movement of Antigen-Containing Signaling Microclusters toward the Central Region for Antigen Gathering by B Cells

(A) Western blot of A20 B cells expressing dynamitin-GFP.

(B and C) A20 B cells transiently transfected with DYNC1H1shRNA (also encoding IRES-GFP) were sorted according to GFP expression, and (B) cells were re-examined for GFP expression after 2 days by flow cytometry.

(C) Western blot of sorted populations for DYNC1H1 expression, quantified over three independent experiments. Mean ± SEM.

(D and E) A20 B cells expressing dynamitin-GFP or DYNC1H1 shRNA were settled on lipid bilayers containing anti-κ (Ag). TIRFM images (D) tracking Ag (E) microcluster movement and net movement of Ag microclusters between 4–14 min toward cell center are shown. Mean ± SEM. Data are from at least 400 microcluster tracks over two independent experiments. The scale bar represents 5 μm. Student's t test: ***$p < 0.0001$. See also Figure S5 and Movie S7.

recruited to and moved with antigen microclusters toward the center in WT DT40 B cells, similar to our earlier observations in primary murine cells (Figures 4C and 4D). Importantly, and in contrast, dynein localization to antigen microclusters was dramatically impaired in *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, and *Cbl*$^{-/-}$ B cells (Figure 7B; Movie S8). To quantify the dynamics of dynein localization, we generated "masks" corresponding to antigen microcluster tracks and measured the intensity of DYNC1I1-GFP fluorescence in these tracks (see Supplemental Experimental Procedures). This quantification demonstrated that dynein localization was significantly reduced in *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, and *Cbl*$^{-/-}$ cells (Figures 7B and 7C). This reduced recruitment and/or retention of dynein provides a reasonable explanation as to why antigen microcluster movement is abrogated in the absence of the three cellular mediators. Thus, it seems likely that a critical threshold of dynein recruitment and/or retention at the microcluster must be surpassed in order to mediate efficient linkage and transport of microclusters on the microtubule network.

Overall, we have identified a role for dynein in the movement of BCR microclusters along the microtubule network. Because antigen accumulation is required for antigen internalization, we have established a critical role for the microtubule network in mediating B cell activation.

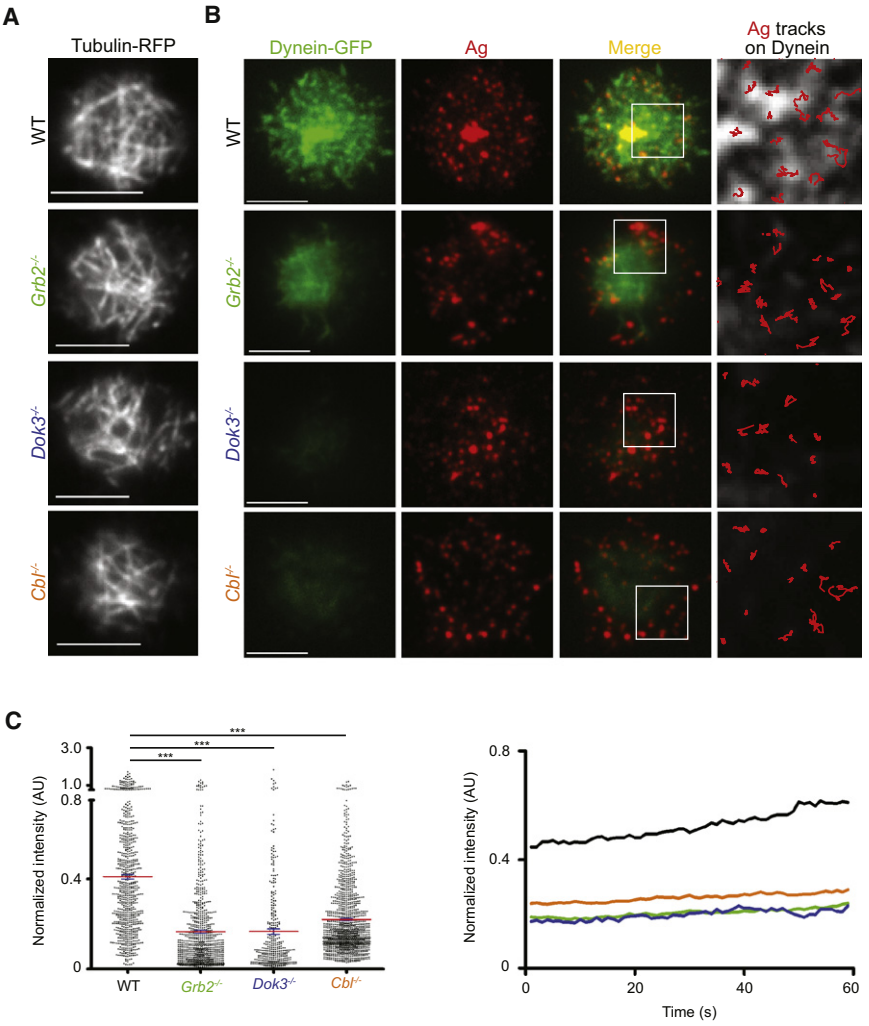

Figure 7. Dynein Localization to the BCR Microcluster Is Required for Antigen Gathering

(A–C) DT40 WT, *Grb2*$^{-/-}$, *Dok3*$^{-/-}$ and *Cbl*$^{-/-}$ B cells were settled on lipid bilayers containing anti-IgM (Ag, red).

(A) TIRFM images of cells expressing α-tubulin-RFP at 5 min.

(B) TIRFM images of cells expressing DYNC1I1-GFP over 1 min collecting one frame s^{-1}. Ag microclusters were tracked and are highlighted in magnified area (white box).

(C) Normalized Dynein-GFP intensity within Ag tracks (left) with each track as a point at frame one (mean, red; ± SEM, blue) (right) over time. Nonparametric Wilcoxon Mann-Whitney test: ***$p < 0.0001$. The scale bar represents 5 μm. See also Figure S6 and Movie S8.

DISCUSSION

Here, we demonstrate that Grb2, Dok-3, and Cbl, although dispensable for BCR microcluster formation and B cell spreading, are required for antigen gathering during B cell activation. Through a combination of high-resolution microscopy, genetics, and mass spectrometry, we show that these three mediators are required for recruitment of dynein to BCR microclusters and their subsequent movement on microtubules to gather antigen. Because the amount of antigen gathered determines the extent of antigen presentation to T cells (Fleire et al., 2006), it appears that dynein and the microtubule network play an unexpected but central role, alongside Grb2, Dok-3, and Cbl, in B cell activation.

From a panel of DT40 B cells, we had previously identified a critical role for Lyn, Syk, PLCγ2, and Vav in actin-dependent B cell spreading and the amplification of BCR signaling through microclusters (Weber et al., 2008). Here, we carried out an alternative functional screen, revealing a role for Grb2, Dok-3, and Cbl in the process of antigen gathering and concomitant B cell contraction. Using TIRFM, we found that these mediators were recruited to antigen-containing BCR microclusters and that Grb2 itself was critical for this recruitment. Although the ITT motif in the tails of IgG and IgE has been reported to mediate recruitment of Grb2 to the BCR (Engels et al., 2009), it is not clear how this occurs in BCRs lacking the ITT motif, though this could be in part due to an interaction with SLP-65 (Neumann et al., 2009). We found that Grb2 recruitment to the BCR microcluster was dependent on the SH2 domain and required B cell signaling. Because Grb2-deficient mice exhibit an early embryo lethal phenotype, we have performed these analyses in DT40 chicken B cells. One earlier investigation found that Grb2 negatively regulates calcium signaling in B cells and that this inhibition is restrained by a phosphorylation dependent interaction with the adaptor NTAL (Stork et al., 2004). Subsequently, it was found that Grb2 mediates its inhibitory calcium function through an interaction with Dok-3 localized at the plasma membrane (Stork et al., 2007). Interestingly, Dok-3 is highly expressed in several hematopoietic cells including B cells, although it is absent from most T cells (Lemay et al., 2000). Furthermore, Dok-3-deficient mice exhibit enhanced humoral immune responses and B cells from these animals are hyperreactive (Ng et al., 2007), potentially as a result of impaired downregulation of BCR from the cell surface. It has been suggested that Dok-3 targets Grb2 to a Btk-containing membrane compartment, resulting in diminished activation of PLCγ2 and calcium signaling (Stork et al., 2007). However, though informative, previous investigations provided no insight into the role and subcellular localization of Grb2 and Dok-3 after stimulation with antigen on a membrane surface as probably occurs in vivo. Consistent with the previous suggestion, we observed that recruitment of Grb2 to the microcluster is altered in the absence of Dok-3, and in addition here we show that Grb2 also plays a pivotal role in the recruitment of Dok-3 to the microcluster. However, the combination of cooperative interactions that shape the dynamics and stability of the Grb2-,

Dok-3-, and Cbl-containing complex at the microcluster remain unclear and will be the subject of future investigations.

Our observation that the E3 ubiquitin ligase Cbl is recruited to BCR microclusters and participates in antigen gathering in B cells is particularly interesting. Although the two Cbl family members Cbl and Cbl-b are widely held to regulate signaling in lymphocytes (Liu and Gu, 2002), their precise role in BCR-mediated signaling remains controversial. As such, genetic and biochemical evidence suggests that Cbl family members function both as negative and positive regulators of BCR signaling through ubiquitination of Syk and/or calcium signaling via PLCγ2 (Sohn et al., 2003; Yasuda et al., 2002). This apparently conflicting data might result from the different origin or differentiation state of B cells used and potentially the partial redundancy of Cbl family members. One recent study, using the same Cbl-deficient DT40 B cells used here, found delayed BCR internalization dependent on the ubiquitin ligase activity of Cbl at early times after antigen stimulation (Jacob et al., 2008). Indeed, on the basis of observations in mice specifically lacking Cbl and Cbl-b in B cells, it was suggested that Cbl proteins play a critical role in BCR downmodulation through ubiquitination of BCR signalosome components including Syk (Kitaura et al., 2007). Interestingly, our observation that Cbl is required for B cell antigen gathering nicely complements the recent finding that ubiquitination is required for central TCR cluster formation after T cell stimulation (Vardhana et al., 2010). In this work, Dustin and colleagues identified a role for TSG101, the ubiquitin-recognizing component of the endosomal-sorting complex required for transport II, in the downregulation of TCR signaling. The authors postulate that TCR ubiquitination, potentially mediated by ligases such as Cbl, after stimulation with strong agonists allows sorting of TCR into specific degradative sites in the central cluster. Likewise in B cells, Cbl may mediate ubiquitination of the BCR microcluster to allow formation of the central antigen-rich region. However, unlike T cells, gathering of antigen in B cells is not only important for attenuation of signaling but also for the acquisition of antigen and presentation necessary for maximal activation.

Using a combination of SILAC mass spectrometry, biochemistry, and imaging, we reveal that signaling BCR microclusters interact with dynein. Dynein is a homodimer of heavy chains in complex with several noncatalytic subunits and mediates the active minus-end-directed movement of intracellular cargo along microtubules toward the MTOC (reviewed in Kardon and Vale, 2009). We found that localization of dynein at the BCR microcluster is dependent on Grb2, Dok-3, and Cbl and is required for BCR microcluster movement for antigen gathering. However, given that we and others were unable to consistently detect direct interactions between Grb2 and dynein (Neumann et al., 2009), it seems likely that this interaction is potentially indirect or highly dynamic. Although a role for dynein during B cell activation has not been previously reported, dynein is recruited to a ring at the T cell immunological synapse (IS) in a mechanism dependent on SLP-76 and ADAP (Combs et al., 2006; Martín-Cófreces et al., 2008). Importantly, we observed that either the downregulation of dynein expression through RNAi or the overexpression of dynamitin significantly impaired the movement of antigen microclusters. The inhibition of movement was surprisingly dramatic particularly given the residual levels of dynein expression after RNAi treatment; however, because it appears likely that dynein molecules often function in concert to mediate movement (McGrath, 2005), this may be due to a requirement for catalytic cycling to mediate effective recruitment and/or retention of dynein at the BCR microcluster.

On the basis of the interaction between BCR microclusters and dynein, we examined the role of the microtubule network in antigen gathering and visualized BCR microclusters localized to and moving on microtubules in stimulated B cells. However, we cannot definitively establish whether this transport on microtubules is intracellular or extracellular. The B cell microtubule network has remained poorly characterized to date. However, a role for the network during T cell activation is implied by repositioning of the MTOC in response to TCR signaling (Lowin-Kropf et al., 1998) and the localized generation of DAG by PLC-γ (Quann et al., 2009). Interestingly, it has been shown that the histone deacetylase HDAC6, capable of deacetylating α-tubulin, is also required for MTOC translocation and IS formation in T cells in response to antigen (Serrador et al., 2004). This is particularly intriguing given that HDAC6 associates with dynein during microtubule-mediated transport of ubiquitinated proteins to the aggresome (Kawaguchi et al., 2003). However, a role of ubiquitin-binding adaptors such as HDAC6 in B cell activation has not been shown to date.

The microtubule network and dynein participate in the movement of BCR microclusters and antigen gathering, but not in the initial phases of B cell activation. Until now it was assumed that antigen gathering, as in T cells, occurred through passive retrograde actin flow (Kaizuka et al., 2007) or myosin IIA-mediated transport along actin filaments (Ilani et al., 2009). However, whereas sustained signaling in T cells is dependent on the continual generation of TCR microclusters, BCR microclusters are rapidly gathered concomitant with contraction to enable antigen internalization and activation. As such, the relative contribution of the microtubule network and actin cytoskeleton in microcluster movement may differ between B and T cells, and therefore it is possible that the actin cytoskeleton and potentially myosin IIA participate in the early movement of BCR microclusters. It remains unclear how a defect in microcluster movement toward the central region would lead to impaired B cell contraction. Given the evidence for the coupling of the actomyosin and microtubule networks (Salmon et al., 2002), it is possible that alteration of dynein or microtubule dynamics leads to a defect in the actomyosin network thus impairing cellular retraction. Interestingly, the linker protein ezrin and its binding partner Dlg1 have recently been implicated in coordinating the various cytoskeleton reorganizations during T cell activation (Lasserre et al., 2010). In this study, Alcover and colleagues showed that although ezrin accumulates early in F-actin rich regions of the T cell IS, it is also required for organization of the microtubule network and movement of TCR microclusters. However, in contrast to our findings with Grb2, Dok-3, and Cbl, silencing of ezrin was found to alter actin-mediated T cell spreading as well as movement of TCR microclusters. In the light of our current findings, we anticipate that the actin cytoskeleton and the microtubule network cooperate to organize the early and later events of B cell activation, respectively.

On the basis of our results, we propose the following model: In response to antigen, signaling is initiated by Lyn and Syk concomitant with the formation of BCR microclusters and is followed by recruitment of other mediators including Vav and PLC-γ2 to mediate reorganizations of the actin cytoskeleton necessary for B cell spreading (Weber et al., 2008). In addition, BCR microclusters also recruit Grb2, Dok-3, and Cbl, allowing the efficient localization of dynein and movement on microtubules after spreading. Because Cbl mediates ubiquitination of BCR microcluster components including Syk, we suggest that this allows association with adaptors such as HDAC6, marking the microcluster for internalization. This type of microtubule-mediated transport mechanism is analogous to that seen in the transport of ubiquitinated proteins to the aggresome for destruction (Valenzuela-Fernández et al., 2008). Because the amount of antigen gathered determines the extent of antigen presentation to T cells, this is particularly significant in shaping the outcome of B cell activation. Interestingly, although the role of Cbl in BCR internalization has remained unclear, a similar role for Cbl in the endocytosis of growth factor receptors has been established (Petrelli et al., 2002). Thus, this may represent a mechanism to couple movement and internalization of numerous cell surface receptors in biology. Indeed, the accompanying manuscript by Hashimoto-Tane et al. (2011) in this issue of *Immunity* identifies a corresponding role for dynein in the movement of TCR microclusters. Nonetheless, many of the molecular details underlying the process of movement and endocytosis are yet to be established and remain the challenge of future work.

EXPERIMENTAL PROCEDURES

Cell Preparation and Culture

Splenic naive mouse B cells were purified by negative selection resulting in enrichment to 95%–98% B cells as described (Carrasco et al., 2004). Murine B cells were maintained at 37°C in complete medium (RPMI containing 10% FCS, 10 mM HEPES, GlutaMAX, penicillin and streptomycin (all Invitrogen), and 50 μM β-mercaptoethanol (Sigma-Aldrich). All experiments were approved by the Cancer Research UK Animal Ethics Committee and the United Kingdom Home Office. WT, *Grb2*$^{-/-}$, *Dok3*$^{-/-}$, and *Cbl*$^{-/-}$ DT40 B cells including stable transfectants were maintained at 39.5°C in complete medium supplemented with 1% chicken serum (Invitrogen). For microtubule disruption, cells were incubated with 59 μM taxol for 80 min or 10 μg/ml Nocodazole for 15 min (both Sigma) at 37°C.

Planar Lipid Bilayers

Planar lipid bilayers containing various antigens were prepared in FCS2 chambers (Bioptechs) as described (Carrasco et al., 2004). Alexa 633-streptavidin (Molecular Probes) was added to bilayers for loading of mono-biotinylated anti-mouse κ lightchain (clone HB-58) and anti-chicken IgM (clone M1) as antigen.

Microscopy

All confocal, DIC, and IRM images were acquired on an Axiovert LSM 510 microscope (Carl Zeiss) as described (Fleire et al., 2006). TIRF images were acquired with a CCD camera (Cascade II; Photometrics) coupled to a TIRFM system (Cell R; Olympus) with iFLEX2000 640 nm laser (Qioptiq). Dual-View TIRFM recording was achieved as described (Treanor et al., 2010). SIM was carried out with a DeltaVisionOMX microscope (Applied Precision) on A20 or DT40 B cells spread on glass coated with surrogate antigen (anti-mouse IgM Fab or anti-chicken IgM M4) and embedded in low-melting agarose after 5 min. Images were recorded with Cell R (Olympus), Image-Pro (MediaCybernetics), or softWoRx (Applied Precision) and analyzed with Volocity (Improvision), Imaris (Bitplane), and ImageJ (NIH) (for more details, see Supplemental Experimental Procedures).

Constructs and Transfections

TagRFP-α-Tubulin (Tubulin-RFP) and dynein intermediate chain of 74kD-GFP (DYNC1I1-GFP) in the pMSCV-p2-RV vectors were provided by M. Huse. Cbl-YFP was provided by L. Samelson. Dynamitin-GFP (Döhner et al., 2002) and shRNA constructs targeting DYNC1H1 (Tsai et al., 2007) were gifts from R. Vallee. Grb2 and Dok3-GFP constructs have been described (Stork et al., 2007), and A20 or DT40 B cell transfections were carried out as described (Weber et al., 2008). Purified primary murine B cells were incubated with 25 μg/ml LPS for 36 hr before transfection with virus particles generated by packaging retroviral vectors in the Plat-E cell line using 293 Fectin (Invitrogen).

Affinity Purification of Syk from SILAC-Labeled Cells and Mass Spectrometry Analysis

Syk-deficient DT40 B cells expressing STrEP-One tagged Syk were cultured in "heavy" SILAC medium (containing $^{13}C^{15}N$-Lysine and $^{13}C^{15}N$-Arginine) and WT DT40 B cells were grown in "light" unlabeled SILAC medium. After BCR stimulation, lysates of 2×10^8 cells were affinity purified as described (Oellerich et al., 2009), with the exception that eluates were pooled (1:1 ratio) after bead washing. Pooled samples were concentrated, separated, and digested with trypsin and extracted peptides were analyzed by LC-MS-MS on an Orbitrap XL ESI spectrometer (ThermoFisherScientific). Proteins were identified in the database and quantified with Mascot and MaxQuant software (Cox et al., 2009) as described (Neumann et al., 2009).

Immunoprecipitations

A total of 4×10^6 DT40 B cells expressing Citrine-Syk, Grb2-GFP, or Dok3-GFP were pre-equilibrated in IMDM at 37°C and then stimulated for three minutes with 5 μg/ml of an anti-chicken IgM (M4), prior to lysis with 1% NP40, 5 mM EDTA, 150 mM NaCl, 20 mM Tris (pH 8), protease inhibitors (Roche), and 1 μg/ml nocodazole. Cleared lysates were immunoprecipitated with anti-GFP (Abcam and Roche) and protein G Dynabeads (Invitrogen) or protein A/G agarose (SCBT) for 16 hr at 4°C. Beads were washed and resuspended in LDS sample buffer (Invitrogen). Samples were analyzed by LC-MS-MS or SDS-PAGE and then immunoblotted with antibodies against GFP (Roche), DYNC1I1 (Abcam), Cbl, and DCTN1 (both BD Biosciences).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight movies and can be found with this article online at doi:10.1016/j.immuni.2011.06.001.

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REFERENCES

Batista, F.D., Iber, D., and Neuberger, M.S. (2001). B cells acquire antigen from target cells after synapse formation. Nature *411*, 489–494.

Carrasco, Y.R., Fleire, S.J., Cameron, T., Dustin, M.L., and Batista, F.D. (2004). LFA-1/ICAM-1 interaction lowers the threshold of B cell activation by facilitating B cell adhesion and synapse formation. Immunity *20*, 589–599.

Combs, J., Kim, S.J., Tan, S., Ligon, L.A., Holzbaur, E.L.F., Kuhn, J., and Poenie, M. (2006). Recruitment of dynein to the Jurkat immunological synapse. Proc. Natl. Acad. Sci. USA *103*, 14883–14888.

Cox, J., Matic, I., Hilger, M., Nagaraj, N., Selbach, M., Olsen, J.V., and Mann, M. (2009). A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. Nat. Protoc. *4*, 698–705.

Depoil, D., Fleire, S., Treanor, B.L., Weber, M., Harwood, N.E., Marchbank, K.L., Tybulewicz, V.L., and Batista, F.D. (2008). CD19 is essential for B cell activation by promoting B cell receptor-antigen microcluster formation in response to membrane-bound ligand. Nat. Immunol. *9*, 63–72.

Döhner, K., Wolfstein, A., Prank, U., Echeverri, C., Dujardin, D., Vallee, R., and Sodeik, B. (2002). Function of dynein and dynactin in herpes simplex virus capsid transport. Mol. Biol. Cell *13*, 2795–2809.

Engels, N., König, L.M., Heemann, C., Lutz, J., Tsubata, T., Griep, S., Schrader, V., and Wienands, J. (2009). Recruitment of the cytoplasmic adaptor Grb2 to surface IgG and IgE provides antigen receptor-intrinsic costimulation to class-switched B cells. Nat. Immunol. *10*, 1018–1025.

Fleire, S.J., Goldman, J.P., Carrasco, Y.R., Weber, M., Bray, D., and Batista, F.D. (2006). B cell ligand discrimination through a spreading and contraction response. Science *312*, 738–741.

Gustafsson, M.G., Shao, L., Carlton, P.M., Wang, C.J., Golubovskaya, I.N., Cande, W.Z., Agard, D.A., and Sedat, J.W. (2008). Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination. Biophys. J. *94*, 4957–4970.

Hashimoto-Tane, A., Yokosuka, T., Sakata-Sogawa, K., Sakuma, M., Ishihara, C., Tokunaga, M., and Saito, T. (2011). Dynein-driven transport of T CELL receptor microclusters forms the central supramolecular activation cluster and regulates T cell activation. Immunity *34*, this issue, 919–931.

Ilani, T., Vasiliver-Shamis, G., Vardhana, S., Bretscher, A., and Dustin, M.L. (2009). T cell antigen receptor signaling and immunological synapse stability require myosin IIA. Nat. Immunol. *10*, 531–539.

Jacob, M., Todd, L., Sampson, M.F., and Puré, E. (2008). Dual role of Cbl links critical events in BCR endocytosis. Int. Immunol. *20*, 485–497.

Kaizuka, Y., Douglass, A.D., Varma, R., Dustin, M.L., and Vale, R.D. (2007). Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. Proc. Natl. Acad. Sci. USA *104*, 20296–20301.

Kardon, J.R., and Vale, R.D. (2009). Regulators of the cytoplasmic dynein motor. Nat. Rev. Mol. Cell Biol. *10*, 854–865.

Kawaguchi, Y., Kovacs, J.J., McLaurin, A., Vance, J.M., Ito, A., and Yao, T.P. (2003). The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell *115*, 727–738.

Kitaura, Y., Jang, I.K., Wang, Y., Han, Y.-C., Inazu, T., Cadera, E.J., Schlissel, M., Hardy, R.R., and Gu, H. (2007). Control of the B cell-intrinsic tolerance programs by ubiquitin ligases Cbl and Cbl-b. Immunity *26*, 567–578.

Kurosaki, T. (1999). Genetic analysis of B cell antigen receptor signaling. Annu. Rev. Immunol. *17*, 555–592.

Lanzavecchia, A. (1985). Antigen-specific interaction between T and B cells. Nature *314*, 537–539.

Lasserre, R., Charrin, S., Cuche, C., Danckaert, A., Thoulouze, M.-I., de Chaumont, F., Duong, T., Perrault, N., Varin-Blank, N., Olivo-Marin, J.-C., et al. (2010). Ezrin tunes T-cell activation by controlling Dlg1 and microtubule positioning at the immunological synapse. EMBO J. *29*, 2301–2314.

Lemay, S., Davidson, D., Latour, S., and Veillette, A. (2000). Dok-3, a novel adapter molecule involved in the negative regulation of immunoreceptor signaling. Mol. Cell. Biol. *20*, 2743–2754.

Liu, Y.-C., and Gu, H. (2002). Cbl and Cbl-b in T-cell regulation. Trends Immunol. *23*, 140–143.

Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., Bar-Sagi, D., and Schlessinger, J. (1992). The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell *70*, 431–442.

Lowin-Kropf, B., Shapiro, V.S., and Weiss, A. (1998). Cytoskeletal polarization of T cells is regulated by an immunoreceptor tyrosine-based activation motif-dependent mechanism. J. Cell Biol. *140*, 861–871.

Martín-Cófreces, N.B., Robles-Valero, J., Cabrero, J.R., Mittelbrunn, M., Gordón-Alonso, M., Sung, C.-H., Alarcón, B., Vázquez, J., and Sánchez-Madrid, F. (2008). MTOC translocation modulates IS formation and controls sustained T cell signaling. J. Cell Biol. *182*, 951–962.

McGrath, J.L. (2005). Dynein motility: Four heads are better than two. Curr. Biol. *15*, R970–R972.

Monks, C.R., Freiberg, B.A., Kupfer, H., Sciaky, N., and Kupfer, A. (1998). Three-dimensional segregation of supramolecular activation clusters in T cells. Nature *395*, 82–86.

Neumann, K., Oellerich, T., Urlaub, H., and Wienands, J. (2009). The B-lymphoid Grb2 interaction code. Immunol. Rev. *232*, 135–149.

Ng, C.-H., Xu, S., and Lam, K.-P. (2007). Dok-3 plays a nonredundant role in negative regulation of B-cell activation. Blood *110*, 259–266.

Oellerich, T., Grønborg, M., Neumann, K., Hsiao, H.-H., Urlaub, H., and Wienands, J. (2009). SLP-65 phosphorylation dynamics reveals a functional basis for signal integration by receptor-proximal adaptor proteins. Mol. Cell. Proteomics *8*, 1738–1750.

Panchamoorthy, G., Fukazawa, T., Miyake, S., Soltoff, S., Reedquist, K., Druker, B., Shoelson, S., Cantley, L., and Band, H. (1996). p120cbl is a major substrate of tyrosine phosphorylation upon B cell antigen receptor stimulation and interacts in vivo with Fyn and Syk tyrosine kinases, Grb2 and Shc adaptors, and the p85 subunit of phosphatidylinositol 3-kinase. J. Biol. Chem. *271*, 3187–3194.

Peters, J.D., Furlong, M.T., Asai, D.J., Harrison, M.L., and Geahlen, R.L. (1996). Syk, activated by cross-linking the B-cell antigen receptor, localizes to the cytosol where it interacts with and phosphorylates alpha-tubulin on tyrosine. J. Biol. Chem. *271*, 4755–4762.

Petrelli, A., Gilestro, G.F., Lanzardo, S., Comoglio, P.M., Migone, N., and Giordano, S. (2002). The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. Nature *416*, 187–190.

Quann, E.J., Merino, E., Furuta, T., and Huse, M. (2009). Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. Nat. Immunol. *10*, 627–635.

Rajewsky, K. (1996). Clonal selection and learning in the antibody system. Nature *381*, 751–758.

Riedl, J., Crevenna, A.H., Kessenbrock, K., Yu, J.H., Neukirchen, D., Bista, M., Bradke, F., Jenne, D., Holak, T.A., Werb, Z., et al. (2008). Lifeact: A versatile marker to visualize F-actin. Nat. Methods *5*, 605–607.

Salmon, W.C., Adams, M.C., and Waterman-Storer, C.M. (2002). Dual-wavelength fluorescent speckle microscopy reveals coupling of microtubule and actin movements in migrating cells. J. Cell Biol. *158*, 31–37.

Serrador, J.M., Cabrero, J.R., Sancho, D., Mittelbrunn, M., Urzainqui, A., and Sánchez-Madrid, F. (2004). HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. Immunity *20*, 417–428.

Shinohara, H., and Kurosaki, T. (2006). Genetic analysis of B cell signaling. Subcell. Biochem. *40*, 145–187.

Sohn, H.W., Gu, H., and Pierce, S.K. (2003). Cbl-b negatively regulates B cell antigen receptor signaling in mature B cells through ubiquitination of the tyrosine kinase Syk. J. Exp. Med. *197*, 1511–1524.

Stork, B., Engelke, M., Frey, J., Horejsí, V., Hamm-Baarke, A., Schraven, B., Kurosaki, T., and Wienands, J. (2004). Grb2 and the non-T cell activation linker NTAL constitute a Ca(2+)-regulating signal circuit in B lymphocytes. Immunity *21*, 681–691.

Stork, B., Neumann, K., Goldbeck, I., Alers, S., Kähne, T., Naumann, M., Engelke, M., and Wienands, J. (2007). Subcellular localization of Grb2 by the adaptor protein Dok-3 restricts the intensity of Ca2+ signaling in B cells. EMBO J. *26*, 1140–1149.

Tezuka, T., Umemori, H., Fusaki, N., Yagi, T., Takata, M., Kurosaki, T., and Yamamoto, T. (1996). Physical and functional association of the cbl protooncogen product with an src-family protein tyrosine kinase, p53/56lyn, in the B cell antigen receptor-mediated signaling. J. Exp. Med. *183*, 675–680.

Tolar, P., Sohn, H.W., and Pierce, S.K. (2005). The initiation of antigen-induced B cell antigen receptor signaling viewed in living cells by fluorescence resonance energy transfer. Nat. Immunol. *6*, 1168–1176.

Tolar, P., Hanna, J., Krueger, P.D., and Pierce, S.K. (2009). The constant region of the membrane immunoglobulin mediates B cell-receptor clustering and signaling in response to membrane antigens. Immunity *30*, 44–55.

Treanor, B., Depoil, D., Gonzalez-Granja, A., Barral, P., Weber, M., Dushek, O., Bruckbauer, A., and Batista, F.D. (2010). The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor. Immunity *32*, 187–199.

Tsai, J.-W., Bremner, K.H., and Vallee, R.B. (2007). Dual subcellular roles for LIS1 and dynein in radial neuronal migration in live brain tissue. Nat. Neurosci. *10*, 970–979.

Valenzuela-Fernández, A., Cabrero, J.R., Serrador, J.M., and Sánchez-Madrid, F. (2008). HDAC6: A key regulator of cytoskeleton, cell migration and cell-cell interactions. Trends Cell Biol. *18*, 291–297.

Vardhana, S., Choudhuri, K., Varma, R., and Dustin, M.L. (2010). Essential role of ubiquitin and TSG101 protein in formation and function of the central supramolecular activation cluster. Immunity *32*, 531–540.

Varma, R., Campi, G., Yokosuka, T., Saito, T., and Dustin, M.L. (2006). T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. Immunity *25*, 117–127.

Vascotto, F., Lankar, D., Faure-André, G., Vargas, P., Diaz, J., Le Roux, D., Yuseff, M.-I., Sibarita, J.-B., Boes, M., Raposo, G., et al. (2007). The actin-based motor protein myosin II regulates MHC class II trafficking and BCR-driven antigen presentation. J. Cell Biol. *176*, 1007–1019.

Weber, M., Treanor, B., Depoil, D., Shinohara, H., Harwood, N.E., Hikida, M., Kurosaki, T., and Batista, F.D. (2008). Phospholipase C-γ2 and Vav cooperate within signaling microclusters to propagate B cell spreading in response to membrane-bound antigen. J. Exp. Med. *205*, 853–868.

Yang, J., and Reth, M. (2010). Oligomeric organization of the B-cell antigen receptor on resting cells. Nature *467*, 465–469.

Yasuda, T., Maeda, A., Kurosaki, M., Tezuka, T., Hironaka, K., Yamamoto, T., and Kurosaki, T. (2000). Cbl suppresses B cell receptor-mediated phospholipase C (PLC)-γ2 activation by regulating B cell linker protein-PLC-γ2 binding. J. Exp. Med. *191*, 641–650.

Yasuda, T., Tezuka, T., Maeda, A., Inazu, T., Yamanashi, Y., Gu, H., Kurosaki, T., and Yamamoto, T. (2002). Cbl-b positively regulates Btk-mediated activation of phospholipase C-γ2 in B cells. J. Exp. Med. *196*, 51–63.