Polarized Secretion of Lysosomes at the B Cell Synapse Couples Antigen Extraction to Processing and Presentation

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SUMMARY

Engagement of the B cell receptor (BCR) by surface-tethered antigens (Ag) leads to formation of a synapse that promotes Ag uptake for presentation onto major histocompatibility complex class II (MHCII) molecules. We have highlighted the membrane trafficking and associated molecular mechanisms involved in Ag extraction and processing at the B cell synapse. MHCII-containing lysosomes are recruited to the synapse where they locally undergo exocytosis, allowing synapse acidification and the extracellular release of hydrolases that promote the extraction of the immobilized Ag. Lysosome recruitment and secretion results from the polarization of the microtubule-organizing center (MTOC), which relies on the cell division cycle (Cdc42)-downstream effector, atypical protein kinase C (aPKCζ), aPKCζ is phosphorylated upon BCR engagement, associates to lysosomal vesicles, and is required for their polarized secretion at the B cell synapse. Regulation of B lymphocyte polarity therefore emerges as a central mechanism that couples Ag extraction to Ag processing and presentation.

INTRODUCTION

The activation of B lymphocytes is critical for the onset of the adaptive immune response. In lymph nodes, it is initiated by the engagement of the B cell receptor (BCR) with antigens (Ag) immobilized at the surface of macrophages or follicular or migrating dendritic cells (Batista and Harwood, 2009). The BCR includes a surface immunoglobulin (Ig) responsible for Ag recognition and a signaling module composed of the Igα/Igβ dimer (Cambier et al., 1994; Retz and Wienands, 1997). BCR engagement induces a complex cascade of signaling events that ultimately leads to cell proliferation and initiation of germinal center (GC) development. To complete GC formation, activated B lymphocytes must present BCR-uptaken Ag to primed CD4+ T cells (Mitchison, 2004). Productive Ag presentation relies on the convergence of BCR-Ag complexes with lysosomal-like compartments containing MHCII molecules, hydrolases in charge of Ag degradation, and H2-DM molecules required for peptide loading (Lankan et al., 2002).

In vivo, activation of B cells is mainly achieved by Ag retained at the surface of neighboring cells (Carrasco and Batista, 2007; Junt et al., 2007; Suzuki et al., 2009), leading to the formation of an immune synapse (IS) that resembles the one originally described in T cells (Grakoui et al., 1999; Kupfer et al., 1997). Synapse formation allows sustained BCR signaling (Harwood and Batista, 2008; Tolar et al., 2008) and further promotes the efficient extraction of the immobilized Ag for processing and presentation to T cells (Fleire et al., 2006). Ag extraction at the IS occurs through a two-phase membrane spreading and cell contraction response that is orchestrated by the local reorganization of the actin cytoskeleton and involves the small GTPases Rac1 and Rac2 (Aranza et al., 2008; Brezski and Monroe, 2007; Fleire et al., 2006). Actin reorganization and activation of the motor protein Myosin II are also required for convergence of BCR-internalized Ag with MHCII-containing lysosomal compartments (Le Roux et al., 2007; Vascotto et al., 2007).

In contrast to the actin cytoskeleton, the role of microtubules (MTs) in formation of the B cell synapse and in Ag extraction and processing remains unaddressed. Several studies support their possible involvement: first, MHCII molecules use MTs to move bidirectionally in melanoma cells (Wubbolt et al., 1999); second, by ensuring the targeted delivery of effector molecules, the polarization of the MT organizing center (MTOC) promotes IS formation (Martín-Cóbreces et al., 2008) and the delivery of secretory granules in T cells (Stinchcombe and Griffiths, 2007). These findings led to the proposal that recruitment of the MTOC to a specific area of the plasma membrane allows the local regulation of exocytosis and endocytosis by focusing the...
required molecular machineries (Griffiths et al., 2010). Whether polarized vesicle trafficking and secretion take place at the B cell synapse and contribute to its function in extraction of immobilized Ag has not been investigated so far.

We here show that B cells rapidly polarize their MTOC together with MHCII+ and lysosomal-associated membrane protein (LAMP-1)+ lysosomes toward the BCR-Ag interface, a process that relies on the small GTPase Cdc42 and its downstream effector, atypical protein kinase C zeta (aPKCζ). Polarized lysosomes are locally secreted, allowing the acidification of the B cell synapse and the extracellular release of lysosomal proteases that promote the extraction of immobilized Ag. Accordingly, protease inhibition or impairment of lysosome exocytosis via Cdc42 or aPKC silencing compromises Ag processing and presentation to T lymphocytes. Thus, MTOC-dependent exocytosis of secretory lysosomes at the immune synapse is critical for B lymphocytes to acquire their Ag presentation function.

RESULTS

BCR Engagement with Immobilized Ag Induces the Recruitment of MHCII-LAMP-1+ Lysosomes at the IS

We investigated the role of membrane trafficking in extraction and processing of immobilized Ag at the B cell synapse. Latex beads coated or not with specific BCR ligands were used to mimic Ag presentation to B cells (Batista and Neuberger, 2000). Mouse spleen IgM+IgD+ B cells incubated for 1 hr with anti-IgM-F(ab’)2-coated beads displayed a cluster of MHCII+, H2-DM+, and LAMP-1+ vesicles at the cell-bead interface (Figure 1A). This cluster was not observed in B cells stimulated with nonspecific BCR ligands (fibronectin or anti-IgG-F(ab’)2), indicating that BCR engagement is required to recruit MHCII+ lysosomes to the synapse. Similarly, stimulation of IgG+ mouse B lymphoma cells with anti-IgG-F(ab’)2-coated beads triggered the formation of a LAMP-1+ cluster that appeared after 30 min and formed a ring around the Ag-coated bead, which became more evident after 2 hr (Figures 1B and 1C). Because B lymphoma cells display very high surface MHCII expression, MHCII intracellular distribution cannot be studied in these cells by immunofluorescence. However, cryoimmunoelectron microscopy experiments showed that LAMP-1+ lysosomes at the cell-bead interface (Figure S1A available online). Hence, MHCII+ lysosomes displaying all the characteristics of Ag processing compartments are recruited to the synapse that forms at the contact site between the BCR and Ag-coated beads in both IgG+ and naive IgM+ spleen-derived B lymphocytes. This synaptic structure will be referred to as the IS hereafter.

We next characterized lysosome dynamics at the IS by using total internal reflection fluorescence microscopy (TIRFM) to image the cell juxta-membrane region. IgG+ B lymphoma cells expressing CatD-RFP+ vesicles in B lymphoma cells plated on anti-IgG-coated slides. The white arrow shows vesicle clustering. Time is shown in seconds; scale bar represents 5 μm.

(L) Lysosome number in the subplasmalemmal region increased over time (bars represent mean ± SEM; n = 29, 82, and 60 cells at 10, 30, and 60 min, respectively, p values: 10 versus 30 min, p < 0.0001; 10 versus 60 min, p = 0.0002 (Mann-Whitney).

Figure 1. BCR Engagement with Immobilized Ag Induces the Recruitment of MHCII-LAMP-1+ Lysosomes at the IS

(A and B) Spleen B cells from MHCII-GFP mice (A) or mouse lymphoma IgG+ B cells (B) were incubated with anti-IgM- or anti-IgG-coated beads for 1 hr at 37°C. Cells were stained for LAMP-1 and GFP or for H2-DM and LAMP-1. Scale bars represent 3 μm. Dashed circles indicate bead position.

(C) Kinetics of LAMP-1+ recruitment to the IS in B lymphoma cells interacting with anti-IgG-coated beads. Time is shown in minutes. Scale bars represent 3 μm.

(D) TIRFM images of CatD-RFP+ vesicles in B lymphoma cells plated on anti-IgG-coated slides. The white arrow shows vesicle clustering. Time is shown in seconds; scale bar represents 5 μm.

(E) Lysosome number in the subplasmalemmal region increased over time (bars represent mean ± SEM; n = 29, 82, and 60 cells at 10, 30, and 60 min, respectively, p values: 10 versus 30 min, p < 0.0001; 10 versus 60 min, p = 0.0002 (Mann-Whitney).
a live-cell marker of MHCII⁺ and LAMP-1⁺ vesicles (Figures S1B and S1C). B cells readily spread onto glass slides coated with F(ab)₂ anti-IgG but not with F(ab')₂ anti-IgM (not shown), indicating that such surface appropriately mimicked Ag-presenting membranes. The number of CatD-RFP-labeled lysosomes detected in the evanescent field increased between 10 and 30 min after B cell plating on Ag-coated slides (Figure 1E). Local vesicle clustering and collective movements were observed (Figure 1D, white arrows; Movie S1). Although a minor fraction of lysosomes moved rapidly, most of them displayed slow diffusive movements or were almost immobile, suggesting that they were attached to the plasma membrane. We conclude that engagement of the BCR by surface-tethered Ag triggers the recruitment of lysosomes and their immobilization at the IS.

MTOC Polarization Is Required for Recruitment of Lysosomes to the Immune Synapse

Because MHCII⁺ vesicles have been shown to move on MTs (Wubboldt et al., 1999), we investigated whether polarization of the MT cytoskeleton accounts for the recruitment of MHCII⁺ lysosomes to the IS. Immunofluorescence experiments where α-tubulin was labeled showed that the MHCII⁺-LAMP-1⁺ cluster that formed at the cell-bead contact site was apposed to the MTOC, suggesting that BCR engagement with immobilized Ag induced MTOC polarization (Figures 2A and 2B). Labeling of the MTOC with a γ-tubulin Ab showed that it was indeed polarized to the IS in activated primary and lymphoma B cells (Figures 2C and 2D). A similar observation was made when incubating spleen B cells that express a hen egg lysozyme (HEL)-specific BCR with HEL-coated beads (Figure 2C). MTOC polarization was quantified by calculating a “polarity index” corresponding to the distance between the MTOC and the bead center of mass divided by the distance between both the cell and the bead centers of mass (Figure 2E). Most of the cells incubated with nonactivating beads displayed a polarity index similar or higher than 1, indicating that their MTOC was located near the cell center (Figures 2F and 2G). In contrast, a large proportion of B cells treated with activating beads exhibited a polarity index between 0.5 and 0.75, showing that their MTOC had been positioned toward the IS. Similar results were obtained when monitoring MTOC polarization toward anti-IgG-coated glass slides by TIRFM: when we used a penetration depth of 300 nm, we found that ~70% of the cells had polarized their MTOC toward the immobilized Ag within 30 min after plating (Figure S2).

Time-lapse analysis of B cells purified from transgenic mice expressing the MTOC marker Centrin fused to green fluorescent protein (GFP) showed that BCR engagement triggered dynamic oscillations of the MTOC (Figure 2H; Movie S2). The MTOC moved back and forward, progressively positioning toward the IS, while the polarity index shifted from 1 to 0.6 (Figure 2I). Similar observations were made in B lymphoma cells (Figure 3A; Movie S3). Cotransfection of Centrin-GFP and CatD-RFP in these cells showed that MTOC and lysosome recruitment to the cell-bead interface occurred concomitantly (Figures 3A and 3B). We conclude that BCR engagement triggers the polarization of both lysosomes and the MTOC toward the IS.

To determine whether polarization of the MTOC directs the trafficking of lysosomes to the IS, we analyzed the impact of MTOC disruption on lysosome distribution via two-photon laser ablation. Strikingly, CatD-RFP⁺ vesicles remained dispersed all over the cell cortex and did not cluster at the IS in cells whose MTOC had been ablated (Figure 3C). Indeed, whereas the distance between the bead and lysosome centers of mass decreased in time in nonablated cells, it did not vary in cells whose MTOC had been eliminated (Figure 3D). Importantly, lysosome recruitment and clustering at the cell-bead contact site was not affected when ablating one lysosomal vesicle, demonstrating that the impact of MTOC destruction on lysosome polarization did not result from a bystander effect of laser ablation (Figure 3C). Hence, polarization of the MTOC triggers the directed trafficking of MHCII⁺-LAMP-1⁺ lysosomes to the synapse that forms upon BCR engagement with immobilized Ag.

MTOC and Lysosome Polarization to the Immune Synapse Requires Cdc42

To define the impact of BCR-induced polarity on IS function, we searched for potential regulators. A likely candidate was the small GTPase Cdc42, which is proposed to control the positioning of the MT network in many cell types (Iden and Collard, 2008). Pull-down experiments with p21-activated kinase (PAK1) fused to glutathione S-transferase (GST) protein revealed that Cdc42 was activated upon BCR engagement with immobilized Ag (Figure S3A). Transfection of the Cdc42-N17 dominant-negative form in B lymphoma cells severely compromised the polarization of the MTOC toward the IS (Figure 4A; Figure S3B), whereas no effect was observed when transfecting the Cdc42-V12 constitutive active form. Silencing of Cdc42 with an shRNA-GFP construct that led to a ~50% decrease in its protein expression (Figure S3C) significantly impaired MTOC polarization to the cell-bead interface (Figures 4B and 4C). MTOC polarization was rescued when introducing an shRNA-insensitive Cdc42 construct in silenced cells, excluding off-target effects (Figure S3D). Time-lapse analysis showed that although Cdc42-depleted B lymphoma cells displayed important MTOC oscillations upon contact with the activating bead, they did not lead to its polarization to the IS (Figure S4). Equivalent conclusions were reached in mouse splenic B cells transfected with two different Cdc42-specific siRNAs (Figure S3E): neither their MTOC nor their MHCII-GFP⁺-LAMP-1⁺ compartment was recruited to the cell-bead synaptic interface (Figures 4D and 4E). Quantification of both MTOC and lysosome polarization in single B lymphoma cells showed that lysosomes from Cdc42-silenced cells that displayed a nonpolarized MTOC were not recruited to the IS, even after 2 hr of incubation (Figures 4F and 4G). In agreement with this result, Cdc42 silencing significantly lowered the density of CatD-mRFP⁺ vesicles detected by TIRFM in cells plated on anti-IgG slides (Figure S6B). Together, our data show that Cdc42 is activated upon BCR engagement, an event that is required to polarize the MTOC and drive the recruitment of lysosomes to the IS.

Nonpolarized Cdc42-Silenced B Cells Show Impaired Ag Processing and Presentation

Having shown that B cell polarization needs Cdc42, we used this finding to assess whether MTOC and lysosome recruitment at the IS impacts on the processing of immobilized Ag. B lymphoma cells were incubated with beads coated with specific BCR ligands plus the LACK Ag from Leishmania major. Their
ability to present LACK-derived MHCII-peptide complexes to a specific T cell hybridoma was then measured by monitoring interleukin-2 (IL-2) secretion. Ag presentation was observed only if LACK was coupled to beads coated with specific BCR ligands (anti-IgG+LACK), demonstrating that Ag targeting to the BCR was required for presentation (Figure 5A). Noticeably, expression of Cdc42-N17 or Cdc42 shRNA constructs significantly decreased the presentation of bead-associated LACK to IgM+ spleen B cells (Figure 5B).
Figure 3. MTOC Polarization Directs the Trafficking of Lysosomes toward the IS

(A) Sequential images of a B lymphoma cell expressing CatD-RFP and Centrin-GFP and engaged with an anti-IgG-coated bead (dashed circles). Scale bar represents 3 μm.

(B) The distance between the bead center of mass (CM) and either the MTOC (green dots) or the CM of the CatD-RFP vesicle population (red dots) was plotted in time.

(C) Sequential images of a B lymphoma cell expressing CatD-RFP and Centrin-GFP engaged with anti-IgG-coated beads of an untreated cell (top) and in a cell in which a lysosome (bottom) or the MTOC (middle) was ablated with a 2-photon laser. Arrows show the position of the MTOC in control and lysosome-ablated cells and white circles show ablated regions. Scale bars represent 3 μm.

(D) The relative distance between CatD-RFP lysosomes and the Ag-coated bead was calculated for each condition as in (B).
Figure 4. MTOC and Lysosome Polarization to the IS Rely on Cdc42

(A) WT, constitutive active (V12), or dominant-negative (N17) Cdc42-GFP-expressing B lymphoma cells were incubated with anti-IgG-coated beads for 1 hr and stained for γ-tubulin and LAMP-1. Insets show magnification of the MTOC surrounded by Cdc42-GFP and LAMP-1 vesicles. (B and D) Control and Cdc42-silenced B lymphoma cells that coexpress GFP (B) and MHCII-GFP primary B control (siControl) or Cdc42-silenced (siCdc42) cells (D) were activated as described above and stained for γ-tubulin and LAMP-1. (C and E) Distribution of MTOC polarity indexes observed in control and Cdc42-silenced B lymphoma cells and in IgM⁺ spleen B cells after 1 hr of incubation with Ag-coated beads. p values: shControl versus shCdc42 = 7.63 × 10⁻³; siControl versus siCdc42-A = 3.67 × 10⁻²; siControl versus siCdc42-(A+B) = 1.018 × 10⁻² (KS test). (F) Scheme depicting how MTOC and LAMP-1 polarization toward the bead were measured. (G) shControl and shCdc42 GFP⁺ B lymphoma cells were incubated with anti-IgG-coated beads. Cell-bead conjugates containing GFP-positive cells (GFP not shown) were stained for γ-tubulin and LAMP-1. Graphs in the lower panel show the distance between the MTOC and the bead CM and the percent of LAMP-1 recruited to the bead (n ≥ 20, 4 independent experiments). Scale bars represent 3 μm.
Figure 5. MTOC and Lysosome Polarization to the IS Promotes Ag Presentation

(A and B) Upper panels show Ag (Lack) presentation assays with control and Cdc42-silenced B lymphoma cells (A) or control and Cdc42-silenced spleen B cells (B). Lower panels show the peptide controls for cells used in the Ag presentation assays. Mean amounts of IL-2 ± SD are shown for a representative experiment. p values: shControl versus shCdc42, p = 0.0120; siControl versus siCdc42, p < 0.0001 (t test).

(C) Confocal images of control or Cdc42-silenced B cell lines incubated with anti-IgG-Lack-beads for 4 hr at 37°C. Cells were fixed and stained for LAMP-1 and I-Ad-LACK156-173 complexes. Scale bars represent 5 μm.

(D) Mean fluorescence intensity (MFI) of I-Aδ-LACK156-173 complexes quantified per cell pooled from two independent experiments; p = 0.0002 (t test).

(E) Confocal images of B lymphoma cells overexpressing PLK4 interacting with anti-IgG- or anti-IgM-coated beads for 1 hr. 3D projections of γ-tubulin and LAMP-1 staining are shown; scale bars represent 5 μm. The inset shows a magnification of multiple MTOCs surrounded by LAMP-1+ vesicles at the Ag-bead contact site.

(F) Ag presentation assay for control and PLK4-overexpressing cells. Mean amounts of IL-2 ± SD were obtained by pooling two independent experiments; control versus PLK4, p = 0.0022 (Mann-Whitney test).

(G) Peptide controls for control and PLK4-overexpressing cells.
Figure 6. Exocytosis of Lysosomes Takes Place at the IS and Is Required for Efficient Extraction of Immobilized Ag

(A) Control and Cdc42-silenced GFP⁺ lymphoma B cells (GFP not shown) were incubated with anti-IgG+OVA or anti-IgM+OVA beads for different time points. Fixed cell-bead conjugates were stained for OVA and LAMP-1. Scale bars represent 3 μm.

(B) The OVA amount on beads interacting with GFP⁺ shControl and shCdc42 cells was calculated (see Experimental Procedures). Each bar represents the mean ± SEM percent of OVA relative to time 0 and was obtained from four independent experiments (n > 25). p values: shControl versus shCdc42 at t = 1 hr, p = 0.0015; and at t = 2 hr, p = 0.00054 (t test).

Figure 6 A and 6B). Remarkably, OVA extraction from the bead surface needed BCR engagement coupling OVA to nonactivating beads, indicating that OVA anti-IgG+OVA-beads). This decrease was not observed when at 1–2 hr after incubation with control cells (Figures 6 A and 6B, decrease in the amount of bead-associated OVA was observed at 10%–20% of B cells (Figure S5B). Interestingly, these cells were larger than control cells and their MTOCs were dispersed when incubated with nonactivating particles but clustered toward the beads when these engaged their BCR (Figure 5E). Strikingly, MTOC amplification in 10%–20% of B cells led to a ~30% increased in Ag presentation (Figure 5F), suggesting that the presence of multiple MTOCs promotes Ag presentation. No effect of MTOC amplification on peptide presentation was observed, indicating that the increase in Ag presentation did not merely result from enhanced cell size or surface MHCII expression but rather from improved Ag processing (Figure 5G). Together, these data suggest that the ability of B cells to process and present immobilized Ag is linked to their capacity to polarize in response to BCR stimulation.

**B Cell Polarization Leads to Lysosome Secretion at the Immune Synapse**

We next investigated whether MTOC and lysosome polarization to the IS promotes Ag processing by facilitating the extraction of immobilized Ag. Ag extraction was evaluated by monitoring the disappearance of ovalbumin (OVA) coupled to activating or nonactivating beads by immunofluorescence. A substantial decrease in the amount of bead-associated OVA was observed at 1–2 hr after incubation with control cells (Figures 6A and 6B, anti-IgG+OVA-beads). This decrease was not observed when coupling OVA to nonactivating beads, indicating that OVA extraction from the bead surface needed BCR engagement (Figures 6A and 6B, anti-IgM+OVA-beads). Remarkably, OVA disappearance was significantly reduced in Cdc42-silenced cells. In contrast, BCR-mediated endocytosis of soluble multivalent ligands was not affected in these cells (Figure S5H), showing that the decrease in Ag extraction of Cdc42–silenced cells was not due to a global defect in endocytosis. Given that BCR endocytosis relies on cortical actin (Onabajo et al., 2008; Stoddart et al., 2005), this implies that (1) Cdc42 silencing does not disturb the actin cortex and (2) impaired Ag extraction in Cdc42-silenced cells does not result from defective Ag internalization because of altered actin organization.

We next investigated the mechanisms by which Cdc42-dependent cell polarization regulates the extraction of immobilized Ag. We postulated that lysosomes may undergo exocytosis at the IS, resulting in the local release of proteases that would facilitate Ag extraction. To test this hypothesis, we fused the pH-insensitive CatD-mRFP construct to a super ecliptic pHluorin domain, whose fluorescence is low at acidic pH and steadily increases upon pH elevation (pKa = 7.1; Figure S6C; Puthenveedu et al., 2010; Sankaranarayanan et al., 2000). As expected, lysosomes from B lymphoma cells expressing CatD-mRFP-pHluorin displayed green fluorescence only when exposed to pH-neutralizing drugs (Figure S6D). When these cells were plated on anti-IgG-coated slides and analyzed by TIRFM, their lysosomes were red but displayed low green fluorescence, indicating that their luminal pH had remained acidic (Figure 6C). Noticeably, monitoring the dynamics of these vesicles showed the occasional appearance of green lysosomes, showing that their acidic content had been neutralized most probably upon exocytosis (Figure 6C, see arrows). Green fluorescent signals were transient (~2 s), suggesting that fluorescent CatD diffused after secretion. Signal intensity was rather low, but they were reproducibly observed (~2.4 events/cell/min in control cells; Figure 6C). Not surprisingly, this number decreased to ~0.9 in Cdc42-silenced cells, showing that lysosome exocytosis at the IS relies on Cdc42. Interestingly, lysosome mobility was altered in Cdc42-silenced cells: calculation of diffusion coefficients (Dv) along lysosome trajectories indicated that the probability of lysosome docking (defined as an immobilization period longer than 10 s, see Supplemental Experimental Procedures; Huet et al., 2006; Desnos et al., 2007) was significantly decreased in Cdc42-silenced cells (Figure 6D). Altogether our data suggest that Cdc42 regulates lysosome exocytosis at the IS by promoting their recruitment and by facilitating their local immobilization and fusion with the plasma membrane.
Figure 7. Cdc42-Dependent Activation of aPKCζ Controls MTOC Polarization and MHCII-LAMP-1+ Secretion at the IS

(A) Mouse B lymphoma cells were incubated with anti-IgG- or anti-IgM-coated beads for 1 hr at 37°C ± 20 μM of the aPKCζ pseudo-substrate. Lysates were analyzed by immunoblot for phospho-aPKCζ (aPKC-P) and actin expression.

(B) B lymphoma cells were incubated with anti-IgG or anti-IgM beads for 1 hr at 37°C and stained for aPKC-P and LAMP-1.

(C) Quantification of aPKC-P integrated fluorescence intensity (FI) in cells interacting with anti-IgM beads or anti-IgG beads ± 20 μM of aPKCζ pseudo-substrate. The FI for each cell was normalized to the mean FI of the negative control (anti-IgM beads; MFI = 1); three independent experiments, p < 0.0001 (Mann-Whitney test).

(D) Ag presentation assay with control and aPKCζ-silenced B cells. Mean amounts of IL-2 ± SD were obtained by pooling three independent experiments. p values: shControl versus sh-aPKCζ-A, p < 0.0001; shControl versus sh-aPKCζ-B, p = 0.0009 (t test). The lower panel shows the peptide control for B cells used in a representative experiment.
Lysosome Secretion at the Immune Synapse Is Critical for Efficient Extraction and Processing of Immobilized Ag

Having shown that lysosomes are secreted at the IS, we investigated whether the local release of their proteolytic content contributes to the extraction of the immobilized Ag. To this mean, we added protease inhibitors and/or the alkalizing agent chloroquine to the extracellular milieu prior to B cell activation and evaluated their impact on Ag extraction, as measured by OVA disappearance from activating beads. Chloroquine as well as all protease inhibitors decreased the ability of B cells to extract the Ag, the more effective being the cysteine protease inhibitor DCG-04 (Figure 6H). DCG-04 is non-cell-permeable (Lennon-Duménil et al., 2002) and in B cells was not targeted to endolysosomal compartments by fluid phase endocytosis, as indicated by the fact that it could not be detected in cellular lysates from cells incubated with 10 μM DCG-04 for 1 hr (Figure S6E). Thus, the activity of extracellular proteases is required for efficient extraction of immobilized Ag by B cells.

To verify that the Ag was indeed being proteolyzed extracellularly, we coupled it to Cypher5, a dye whose fluorescence increases at acidic pH (Milasta et al., 2005). We reasoned that, if internalized prior to degradation, Cypher5-labeled Ag should be observed in polarized lysosomes. On the contrary, the lack of fluorescence in lysosomes would indicate that bead-associated Ag are proteolyzed at the IS at first. Strikingly, Ag-coated beads acquired a strong fluorescent signal approximately 30 min upon cell contact (Figure 6E), suggesting that the synaptic pH had become acidic. Accordingly, we observed a rapid disappearance of the green fluorescent signal after addition of chloroquine (Figure 6G). Bead fluorescence was significantly decreased upon Cdc42 silencing (Figure 6F), consistent with inhibition of lysosome secretion. Importantly, local acidification of the extracellular space resulting from lysosome exocytosis might account for the relatively low pH-phluorin signals observed by TIRFM. Labeling of cell-associated activating beads without cell permeabilization showed that bead-associated Ag rapidly became partially or not accessible to Abs because of membrane spreading (Figures S6F and S6G). These findings suggest that the cell–bead interface is a sealed zone that can prevent proton diffusion and allow local pH acidification. Importantly, Cypher5-labeled anti-IgG was not detected in intracellular vesicles, suggesting that it had been proteolyzed at least in part prior to internalization (Figure 6E). We conclude that polarized secretion of lysosomes at the IS allows the local release of hydrolases that contribute to efficient extraction of immobilized Ag having engaged the BCR.

Lysosome Secretion at the Immune Synapse Relies on the Activation of Atypical PKCζ Downstream of Cdc42

We next searched for Cdc42 effector molecules involved in polarized secretion of lysosomes at the B cell IS. Atypical protein kinase C (aPKC) was a good candidate because, together with partitioning defective proteins Par6 and Par3, it forms the Par complex known to control cell polarity downstream of Cdc42 in multiple cell types (Goldstein and Macara, 2007). aPKC autophosphorylates upon interaction with Par6-bound Cdc42-GTP, resulting in Par3 phosphorylation and MTOC polarization (Iden and Collard, 2008). Interestingly, it was shown that aPKC is activated upon BCR stimulation and mice genetically ablated for aPKCζ—the aPKC form expressed in lymphocytes—fail to mount an efficient humoral immune response (Guo et al., 2009; Martin et al., 2002). Immunoblot analysis showed that expression of phospho-aPKCζ strongly increased upon B cell incubation with activating beads (Figure 7A). Phospho-aPKCζ was not observed when incubating B cells with nonactivating beads or when inhibiting its kinase activity, showing the specificity of the phospho-aPKC Ab (Figures 7A–7C). Silencing of aPKCζ with two different shRNA constructs strongly impaired the recruitment of the LAMP-1+ MTOC and lysosome polarization to the IS were impaired in aPKCζ-silenced cells, as shown by a significant increase in polarity indexes as well as in the recruitment of the LAMP-1+ vesicles at the cell–bead interface (Figure 7E; Figure S7C). Similar results were obtained when treating the cells with an aPKC inhibitor (Figure S7D). Time-lapse analysis showed that in aPKCζ-silenced cells, the MTOC and lysosomes had started to polarize toward the IS but that polarity was lost after ~30 min after BCR engagement (Figure 7F). Remarkably, synapse acidification was not observed in these individual cells, as shown by the lack of fluorescent signal from beads coated in anti-IgG-Cypher beads engaged to shControl and sh-aPKCζ. Arrows show the MTOC surrounded by lysosomes.

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with Cypher5-coupled Ag (Figure 7G; Figure S7E), indicating that lysosome secretion at the synapse is compromised in the absence of the enzyme. We conclude that the Cdc42 effector protein aPKCζ is required for MTOC polarization and exocytosis of lysosomes at the B cell synapse.

**DISCUSSION**

Establishment of cell polarity is crucial for many cellular processes, including asymmetric cell division, directed cell migration, and tissue development. We here bring evidence for B lymphocyte polarization in response to Ag stimulation and provide insights on how they use polarity to promote their Ag presentation function. Our results support a role for MTOC polarization in directing the trafficking of MHCII-LAMP-1 vesicles toward the synapse, thereby facilitating their local recruitment and secretion to couple Ag extraction to processing. Cell polarity thus emerges as an essential mechanism for B cells to acquire their Ag presentation function.

BCR-mediated uptake of membrane-tethered Ag relies on the formation of an IS that allows Ag gathering and extraction for presentation to T cells (Batista et al., 2001; Fleire et al., 2006). The precise mechanism used by B cells to extract and process immobilized Ag remains unclear. Our results show that exocytosis of lysosomes at the IS provides an acidic and protease-rich environment that facilitates Ag extraction prior to internalization. This finding is reminiscent of a recent observation made in macrophages where lysosome secretion provides a sealed acidic extracellular zone to favor digestion of aggregated lipoproteins by lipases and proteases (Haka et al., 2009). The model used in our study showed an important role for secreted proteases in Ag extraction, but lysosomal lipases may equally contribute to extraction when synapses form between the BCR and membrane-bound Ag.

In addition to hydrolase secretion, polarized trafficking of lysosomes facilitates the local concentration of MHCII and accessory molecules required for Ag processing. Thus, polarized lysosome secretion couples Ag extraction to formation of the Ag processing compartment, allowing the spatio-temporal coordination of these two events ultimately required for T cell-B cell cooperation. Different nonexclusive scenarios can be envisioned for the nature of the compartment where Ag processing takes place. BCR-bound large Ag chopped by secreted proteases may be directly loaded onto surface MHCII molecules (Moss et al., 2007). Alternatively, lysosome exocytosis might be associated to compensatory endocytic events, allowing the rapid uptake of predigested BCR-Ag complexes, which would be further degraded and loaded onto MHCII molecules intracellularly. This second scenario is consistent with data obtained in T cells, where secreted LAMP-1 vesicles are rapidly retrieved by endocytosis at the IS (Liu et al., 2009). One could speculate that the nature and amount of hydrolases secreted at the B cell synapse define whether the Ag is processed extra- and/or intra-cellularly, determining the size of the peptide Ag to be presented to T cells and the fate of the immune response.

We found that MTOC and lysosome polarization rely on BCR-dependent activation of the small GTPase Cdc42 and its effector protein aPKC. This finding is consistent with observations made in other cell types—from the budding yeast to activated T lymphocytes—where Cdc42 plays a major role in cell polarity (Iden and Collard, 2008). Importantly, genetic evidence for the involvement of both Cdc42 and aPKC in B cell function has been provided: Cdc42 genetic ablation impairs B cell development and aPKCζ-null mice show altered humoral immune responses (Guo et al., 2009; Martin et al., 2002). aPKCζ is activated downstream of Cdc42 and is found in association to lysosomes. Interestingly, although Cdc42- and aPKCζ-silenced B cells display impaired MTOC and lysosome polarization upon BCR engagement, time-lapse analysis showed that they intervene at different stages: although silencing of Cdc42 compromises MTOC and lysosome polarization, their polarization occurs in the absence of aPKCζ but is rapidly lost. These results suggest that Cdc42-GTP interacts with (1) unknown effector(s) that generate the driving force needed for MTOC polarization and (2) aPKC that allows the stabilization of polarized MTOC and lysosomes at the synapse. This aPKC-dependent step of polarity stabilization is required for the docking and secretion of lysosomes at the IS to promote Ag extraction and processing. This model shows that Cdc42 couples lysosome recruitment at the synapse to local exocytosis and is consistent with the role proposed for the IS as a platform for regulated events of polarized trafficking.

What are the Cdc42 effectors regulating MTOC polarization to the IS? Dynein regulates MTOC polarization in migrating fibroblasts downstream of Cdc42 and in concert with myosin II (Gomes et al., 2005). We found that myosin II is activated upon BCR engagement and controls lysosome polarization at the IS (M.-I.Y. and A.-M.L.-D., unpublished data). Time-lapse imaging showed that MTOC polarization occurred concomitantly with phases of actomyosin contraction that stopped once polarity was stably achieved. In addition, Cdc42-silenced B cells displayed uncontrolled cortical contractility, suggesting that myosin II activity is altered in Cdc42-silenced cells. Thus, MTOC polarization to the IS might rely on the concerted action of dynein and myosin II motor proteins acting downstream of Cdc42. Focusing on molecules that regulate the MT-actin crosstalk will provide insights on how Ag extraction and processing are regulated at the B cell synapse.

**EXPERIMENTAL PROCEDURES**

**Mice and Cells**

I-A^b-GFP genetically targeted mice (MHCII-GFP) were provided by H. Ploegh (Whitehead Institute, MIT, MA) (Boes et al., 2002). The transgenic mouse lines, centrin-GFP, and MD4 have been described (Goodnow et al., 1988; Pulecio et al., 2010). The mouse lymphoma cell line IA1.6 (Lankar et al., 2002) and the LMR7.5 T cell hybridoma that recognizes I-A^b-LACK156-173 complexes were cultured as reported (Le Roux et al., 2007; Vasco et al., 2007). Resting mature spleen IgM^-IgD^- B cells were purified as described (Vasco et al., 2007). All mice were housed at the Institut Curie in an accredited specific-pathogen-free colony and were sacrificed in accordance with the guidelines and regulations of the French Veterinary Department.

**Preparation of Ag-Coated Beads**

4 × 10^3 μm latex NH2-beads (Polyscience) were activated with 8% glutaraldehyde for 2 hr at room temperature (RT). Beads were washed with PBS and incubated overnight (O/N) with different ligands: 10 μg/ml of either F(ab')2 goat anti-mouse-IgM or F(ab')2 goat anti-mouse-IgG (MP Biomedicals) or hen egg white lysozyme (HEL) (Sigma) or 1 μg/ml of Fibronectin (FN) (Sigma).

**Stimulation of B Cells and Immunofluorescence Analysis**

Cells were activated with Ag-coated NH2-beads at a 1:1 ratio and plated on poly-L-lysine-coated glass coverslips for different time points at 37°C, fixed...
in 3% paraformaldehyde (PFA) for 10 min at RT, and quenched in 1 mM glycine for 10 min. Fixed cells were incubated with antibodies in PBS-0.2% BSA-0.05% saponin. For detection of I-A<sup>b</sup>-LACK<sub>156-173</sub> complexes, biotinylated 2C44 and streptavidin-546 (Tyramide kit, Invitrogen) were used as previously described (Vascotto et al., 2007). Immunofluorescence images were acquired on a confocal microscope (LSM Axiovert 720; Carl Zeiss Microimaging, Inc.) with a 63× 1.4 NA oil immersion objective.

**Time-Lapse Analysis**

A Nikon Eclipse TE2000-U microscope equipped with a 100× 1.45NA, oil immersion objective, a PIFOC Objective stepper, a Yokogawa CSU22 confocal unit, and a Roper HQ2 CCD camera was used. B cells expressing the plasmids of interest were attached on a poly-L-lysine-coated 35 mm dishes (Fluorodish) at 37°C and images were acquired with Metamorph software.

**Centrosome Ablation**

Cells were plated on poly-L-lysine-coated 35 mm dishes and imaged with a confocal microscope (Zeiss 710 NLO) equipped with an oil immersion lens (63×1.4 OIL DIC II PL APO). Stacks of images were taken every 2 min for 1 hr at 37°C and 5% CO<sub>2</sub>. After acquiring the first image, laser ablation was performed with a pulsed laser (Mai-Tai HP, Spectra Physics) controlled by the Zen2009 software. The laser beam was focused to a region around the centrosome or lysosome, marked by centrin-GFP or CatD-RFP and 2 iterations of 800 nm laser pulse, corresponding to 40 mW were applied. Ablation was monitored by the absence of recovery of the RFP or GFP signal.

**Ag Presentation**

Lack was coupled to glutaraldehyde-activated NH2 beads together with F(ab<sup>2</sup>)<sub>2</sub> anti-mouse-IgM or anti-mouse-Ig2 fragments in equal concentrations. Spleen B cells from BALB/c WT mice or I-A<sup>b</sup> mice (I-A<sup>b</sup>) were incubated with Lack-coated beads or with Lack Peptide for 4 hr at 37°C. B cells were washed and incubated with 0.75 × 10<sup>5</sup> Lack T cell hybridoma for 4 hr. Supernatants from each condition were obtained and IL-2 was measured (BD Bioscience).

**Ag-Extraction Assay**

OVA and F(ab<sup>2</sup>)<sub>2</sub> anti-mouse-IgM or anti-mouse-IgG fragments were coupled to NH2-beads in equal concentrations. Cells incubated in a 1:1 ratio with Ag-coated beads were plated on poly-L-lysine slides for different time points at 37°C, fixed, and stained for OVA with a polyclonal Ab. The amount of OVA present on the beads after each time point was calculated by establishing a fixed area around beads in contact with cells and the fluorescence intensity was measured on 3D projections obtained from the sum of each plane (Metamorph).

**TIRFM**

Cells expressing shRNAs were transfected with plasmids encoding Centrin-GFP, CatD-RFP, or CatD-RFP-phluorin 48 hr before imaging by TIRFM and plated on coverslides (MatTek) coated with anti-IgG-F(ab<sup>2</sup>)<sub>2</sub> or anti-IgM-F(ab<sup>2</sup>)<sub>2</sub> or anti-mouse-IgM-F(ab<sup>2</sup>)<sub>2</sub> 30 min before imaging. TIRF imaging was performed on a custom setup described previously (Huet et al., 2006) with a penetration depth of 150–300 nm. One pixel corresponds to 107.5 nm. Frames were acquired at 7–10 Hz with an exposure time of 100–150 ms. Image segmentation and single-particle tracking were done with multidimensional image analysis software (MIA) as described previously (Desnos et al., 2007). The diffusion coefficient <i>D<sub>PS</sub></i> was calculated along trajectories, immobilization periods were defined as portions of trajectories during which <i>D<sub>PS</sub></i> < 3 × 10<sup>-6</sup> μm<sup>2</sup> s<sup>-1</sup>, and their snapshot occurrence was computed (Desnos et al., 2007).

**Lysosome Exocytosis Visualized by TIRFM**

Cells expressing shRNAs and CatD-RFP-phluorin were plated for 30 min on coverslides coated with ant- IgG-F(ab<sup>2</sup>)<sub>2</sub> and imaged by TIRFM. Cells were selected in the red channel (excitation 514 nm, emission 605–655 nm) and imaged in the green channel (excitation 488 nm, emission 522–548 nm) to detect the transient increases in phluorin fluorescence that follow exocytosis-induced lysosome alkalization. Stacks of 1200 frames were acquired at ~7 Hz with an exposure time of 150 ms. A denoising algorithm was applied (Boulanger et al., 2010) before counting the number of exocytic events.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five movies and can be found with this article online on doi:10.1016/j.immuni.2011.07.008.

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