**ION TRANSPORT**

The channel-kinase TRPM7 regulates antigen gathering and internalization in B cells

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Members of the transient receptor potential (TRP) family of ion channels are cellular sensors involved in numerous physiological and pathological processes. We identified the TRP subfamily M member 7 (TRPM7) channel-kinase as a previously uncharacterized regulator of B cell activation. We showed that TRPM7 played a critical role in the early events of B cell activation through both its ion channel and kinase functions. DT40 B cells deficient in TRPM7 or expressing a kinase-deficient mutant of TRPM7 showed defective gathering of antigen and prolonged B cell receptor (BCR) signaling. We showed that lipid metabolism was altered in TRPM7-deficient cells and in cells expressing a kinase-deficient mutant of TRPM7 and suggest that PLC-γ2 may be a target of the kinase activity of TRPM7. Primary B cells that expressed less TRPM7 or were treated with a pharmacological inhibitor of TRPM7 also displayed defective antigen gathering and increased BCR signaling. Finally, we demonstrated that blocking TRPM7 function compromised antigen internalization and presentation to T cells. These data suggest that TRPM7 controls an essential process required for B cell affinity maturation and the production of high-affinity antibodies.

**INTRODUCTION**

Ion channels play a crucial role in almost every biological process. These critical gateways between the intra- and extracellular environments enable cells to maintain metabolic functions and respond to environmental stimuli. Members of the transient receptor potential (TRP) family have emerged as important cellular sensors for various extracellular stimuli, including pain, temperature, and pressure (1). These ion channels are implicated in both homeostatic and disease processes. In particular, TRP subfamily M member 7 (TRPM7) is an ion channel that regulates cellular magnesium (Mg2+) and calcium (Ca2+) homeostasis (2, 3). TRPM7 is essential for embryonic development because genetic deletion in mice results in embryonic lethality (4, 5) and deletion of TRPM7 from the chicken B cell line DT40 results in growth arrest and cell death (2). Growth arrest can be overcome by culturing cells lacking TRPM7 in medium supplemented with high concentrations of extracellular Mg2+ (3) or partially by overexpression of the Mg2+ transporter MagT1 in TRPM7-deficient DT40 cells (6).

TRPM7 is also notable because it contains both a cation channel and a serine and threonine protein kinase domain (2, 7–9). This suggests that TRPM7 may regulate not only ion movement but also receptor-mediated signal transduction. TRPM7 channel activity is down-regulated by increasing concentrations of intracellular free Mg2+ and Mg2+ nucleotides. TRPM7 ion currents are activated by phospholipase C (PLC)–coupled receptor agonists (10, 11), lending further credence to the idea that TRPM7 may play an important role in receptor-mediated signal transduction.

Despite widespread expression across immune cells, little is known about the role of TRPM7 in the immune response. Specific deletion of TRPM7 in thymocytes results in defective thymopoiesis without altering acute Mg2+ uptake or total cellular Mg2+ abundance (3). TRPM7 is also important for macrophage activation and proliferation, and its activity is differentially regulated in pro- versus anti-inflammatory macrophages (12, 13). In the context of B lymphocytes, relatively little is known except for the aforementioned role in survival of the DT40 B cell line (2, 3). However, the key B cell receptor (BCR) signaling molecule, PLC-γ2, is a substrate for the TRPM7 kinase domain (14). Furthermore, TRPM7 is involved in regulation of the actin cytoskeleton in other cell types, although the importance of the kinase versus channel function of TRPM7 in this aspect is unclear. Overexpression or knockdown of TRPM7 alters cytoskeletal organization, cell migration, and adhesion (10, 15). This may be due to TRPM7 kinase-mediated phosphorylation of nonmuscle myosin IIA (NMMIIA) heavy chain and consequently reduced contractility of the actin cytoskeleton (10). However, a role for the Mg2+ transport function of TRPM7 in the regulation of the actin cytoskeleton has also been proposed, possibly through inhibition of NMMIIA activity by reducing adenosine 5′-diphosphate release rate and affinity for actin (16).

To further understand the role of TRPM7 in the immune response, we investigated the role of TRPM7 in B cell activation. Here, we demonstrated that TRPM7 was important for cytoskeletal integrity in B cells in both the steady state and after antigen stimulation. Moreover, DT40 B cells deficient in TRPM7 or expressing a point mutation in the kinase domain were unable to centrally aggregate antigen after activation. This activity required TRPM7-dependent PLC-γ2 activation and lipid metabolism. Our results were recapitulated in primary B cells expressing only a single allele of TRPM7 or after treatment with a pharmacological inhibitor of TRPM7. Demonstration that antigen internalization was defective in TRPM7-deficient...
cells has important functional consequences for T cell activation. These findings implicate TRPM7 as a previously uncharacterized regulator of B cell activation.

RESULTS

TRPM7 regulates the actin cytoskeleton in B cells

Because TRPM7 regulates actin cytoskeleton rearrangements (10, 15), we examined the effect of TRPM7 deficiency on B cell morphology under steady-state conditions. For these studies, we used the DT40 chicken B cell line, which has been widely used to assess the contribution of various molecules to BCR signaling (18) and the molecular requirements for B cell spreading and microcluster formation (19). When wild-type (WT) and TRPM7 knockout (TRPM7-KO) DT40 B cells were visualized by scanning electron microscopy, we found that WT DT40 cells had numerous short microvilli and filopodia (Fig. 1A), as expected (19). In contrast, the morphology of TRPM7-KO B cells was different (Fig. 1A). Although TRPM7-KO cells had about half the number of cell surface protrusions of WT cells (Fig. 1B), these protrusions were twice as long (Fig. 1C). Similarly, when immunoglobulin M (IgM)–activated WT and TRPM7-KO cells were visualized by scanning electron microscopy, we found that TRPM7-deficient cells spread lamellipodia over a smaller area than did the WT cells but extended elongated filopodia-like surface protrusions (Fig. 1D). These data suggest that TRPM7 is required for specific actin cytoskeletal rearrangements in B cells. Staining these cells for filamentous actin (F-actin) revealed that TRPM7-KO cells spread over a smaller area, and substrate contacts were less circular than those of WT cells (Fig. 1, E to G). Together, these results demonstrate that TRPM7 regulates the actin cytoskeleton in both the steady state and after BCR stimulation.

B cell centralization of antigen is impaired in TRPM7-deficient cells

B cell spreading is accompanied by the formation of BCR-antigen microclusters throughout the contact area with the antigen-bearing membrane (20). This is then followed by a contraction phase during which BCR-antigen microclusters are centrally aggregated (21). Both of these processes are dependent on the actin cytoskeleton because cell spreading and microcluster formation are abrogated when cells are pretreated with actin-depolymerizing agents (21, 22). Similarly, treating B cells with depolymerizing agents after cell spreading abrogates the centripetal movement of microclusters (23, 24) and disrupts microcluster integrity (23). To examine BCR-antigen microcluster formation and dynamics, we used an artificial planar lipid bilayer system in which the surrogate antigen is tethered to the membrane by fluorescently labeled streptavidin. We used total internal reflection fluorescence microscopy (TIRFM) and interference reflection microscopy (IRM) to visualize the spreading of DT40 B cells and their contraction response in this system, which mimics the membrane-bound antigens found in vivo. When activated by membrane-bound antigen, both WT and TRPM7-KO cells spread and formed BCR-antigen microclusters, reaching maximum spreading within 3 min (Fig. 2A and movie S1). TRPM7-KO cells formed microclusters more rapidly and spread over a larger area than did WT cells (Fig. 2, B and C, and fig. S1). IRM images suggested very dynamic membrane ruffling and protrusions in the periphery of WT cells compared to that of TRPM7-deficient cells (movie S1). TRPM7-KO cells did not contract and centrally aggregate antigen as potently as did WT cells and maintained a ~1.5-fold larger mean contact area after 15 min compared to that of WT cells (TRPM7-KO, 58.297 ± 3.146 μm²; WT, 38.584 ± 4.800 μm²) (Fig. 2B and fig. S1). Tracking of individual antigen clusters revealed that antigen microclusters translocated from the periphery to the center of the contact in WT cells, whereas microcluster tracks were more confined to the periphery and did not
translocate to the center of contact in TRPM7-KO cells (Fig. 2D). Consistent with these observations, the mean diffusion coefficient of microclusters was almost three times lower in TRPM7-KO cells than in WT cells (Fig. 2E).

To visualize the actin cytoskeleton during this process, cells were stimulated on antigen–containing bilayers for either 3 or 10 min and then were stained with phalloidin to visualize F-actin by confocal microscopy. Consistent with our live-cell imaging, we found that TRPM7-KO cells rapidly formed microclusters of antigen, which, at 3 min, were larger and less discrete than the microclusters seen in WT cells (Fig. 2F). This was evident by the twofold increase in the total antigen intensity that we observed in TRPM7-KO cells as compared to that in WT cells (Fig. 2G). After 10 min of stimulation, WT cells contracted antigen into a central aggregate that was surrounded by actin (Fig. 2H). Whereas actin was similarly cleared from the contact center in TRPM7-KO cells, the size of the antigen aggregate and consequentely the total amount of antigen accumulated were increased when compared to those of WT cells (Fig. 2I).

Complementation of TRPM7 in TRPM7-KO cells using a doxycycline-inducible human TRPM7 construct (TRPM7-hWT) rescues the growth arrest of TRPM7-KO DT40 cells (3). Using this system, we stimulated WT and TRPM7-hWT DT40 cells that were treated with or without doxycycline and imaged the cells by confocal microscopy. Consistent with our previous findings, uninduced cells aggregated more antigen and failed to contract antigen microclusters more than WT cells (fig. S2, A to D). However, expression of human TRPM7 in TRPM7-KO cells restored the antigen aggregation dynamics to that of WT cells (fig. S2, A to D). These data support our finding that TRPM7 regulates antigen gathering in B cells.

**TRPM7 kinase activity is important for B cell contraction**

To dissect the contribution of the TRPM7 kinase domain, we reconstituted TRPM7-KO cells with a doxycycline-inducible TRPM7-K1648R mutant that lacks phosphotransferase activity (3). Under steady-state conditions, the cellular morphology of B cells expressing this TRPM7 kinase–deficient (TRPM7-KD) mutant was similar to that of TRPM7-deficient cells, with fewer, yet longer, filopodia than those of WT DT40 cells (Fig. 3A). To investigate the role of the kinase domain of TRPM7 during B cell activation, we visualized B cell spreading and the formation of BCR microclusters by TIRFM. Consistent with TRPM7-KO cells, TRPM7-KD cells were unable to contract and form a tight central antigen aggregate, with microclusters persisting for at least 15 min after activation (Fig. 3, B to D; movie S2; and fig. S3). However, TRPM7-KD cells spread more slowly (Fig. 3C) and the number of antigen clusters formed was greater than did TRPM7-KO cells (compare with Fig. 2, A and D). Tracking of individual microclusters revealed that antigen microclusters in TRPM7-KD cells were largely immobilized and there was little translocation to the center of contact (Fig. 3, E and F). The exaggerated phenotype in TRPM7-KD cells may be consistent with a functional coupling between the kinase

**Fig. 2. B cell spreading and the contraction response are impaired in TRPM7-KO cells.** (A to E) TIRFM analysis of WT and TRPM7-KO DT40 B cells stimulated with bilayers containing fluorescently labeled anti-IgM. Images (A) are representative, and the contact area (B) and the number of fluorescent antigen clusters over time (C) are means ± SEM of measurements taken from a minimum of 20 cells. Antigen microclusters tracks from individual cells (D) are representative, and the diffusion coefficient of antigen microclusters (E) is means ± SEM of measurements pooled from three independent experiments. DIC, differential interference contrast. (F to I) Confocal microscopy analysis of actin in WT and TRPM7-KO DT40 B cells after stimulation with lipid bilayers containing anti-IgM for 3 min (F and G) or 10 min (H and I). Images (F and H) are representative. Relative fluorescence intensity (FI) plots indicate the distribution of antigen (pink) and actin (green) along the dashed line. Data on total antigen fluorescence intensity at the contact interface (G and I) are means ± SEM of measurements taken from a minimum of 20 cells. AU, arbitrary units. All images and quantified data are representative of three independent experiments, except for the data in (E). Statistical significance for the data in (B) and (C) was assessed by regression analysis (see fig. S1). Statistical significance for the data in (E), (G), and (I) was assessed by Mann-Whitney test. ***P < 0.001, ****P < 0.0001. Scale bars, 5 μm.
and channel domains (3). Consistent with our live-cell imaging, we found that TRPM7-KD cells spread and formed antigen microclusters within 3 min and showed increased antigen accumulation similar to TRPM7-KO cells by confocal microscopy (Fig. 3, G and H). We observed that BCR microclusters in TRPM7-KD cells stained with phalloidin colocalized with actin (Fig. 3G) to a greater degree than those in either WT or TRPM7-KO cells (Fig. 2F). At 10 min after stimulation, multiple microclusters were still visible in the cell periphery, and there was only a small central accumulation of antigen (Fig. 3I). In contrast to that seen in WT cells, there was a minimal clearing of F-actin (Fig. 3I). These data suggest that the kinase domain of TRPM7 plays an important role in actin organization in B cells. However, the total antigen intensity at this time point was similar to that seen in WT cells (Fig. 3J), which may indicate that perhaps antigen is being internalized, despite the lack of centralization.

B cell spreading and the formation of antigen microclusters are essential to propagate BCR signaling. As microclusters form, several signaling molecules are recruited to form the signalosome, where each microcluster represents a unit of signaling (20). We investigated the effect of TRPM7 on BCR signaling using plate-bound antigen (23) and found that phosphorylation of ERK was increased in both TRPM7-KO and TRPM7-KD cells compared to that in WT cells (Fig. 3, K and L). Together, these results demonstrate an important role for TRPM7 in antigen aggregation and BCR signaling.

**Antigen gathering and BCR signaling cannot be rescued by extracellular Mg$^{2+}$**

Supplementing TRPM7-KO DT40 cells with high levels of extracellular Mg$^{2+}$ is sufficient to rescue their cell survival defect (3). To interrogate whether antigen aggregation and BCR signaling could also be rescued by supplementation with extracellular Mg$^{2+}$, we cultured TRPM7-KO and TRPM7-KD DT40 cells in the presence of 10 mM extracellular Mg$^{2+}$. When cells were activated for 10 min on artificial planar lipid bilayers, we found that Mg$^{2+}$ supplementation had no effect on antigen aggregation in TRPM7-KO or TRPM7-KD cells (fig. S4, A and B). In contrast, supplementation with 10 mM extracellular Mg$^{2+}$ abrogated the increased ERK phosphorylation that we observed in both TRPM7-KO and TRPM7-KD cells (fig. S4, C and D). Thus, increased extracellular Mg$^{2+}$ was sufficient to rescue BCR signaling but not antigen gathering in TRPM7-KO and TRPM7-KD cells.

**Mutation of a TRPM7 phosphorylation site in PLC-γ2 alters BCR-antigen aggregation**

PLC-γ2 is a key BCR signaling molecule that is important in the spreading and...
contracting response (19). The kinase domain of TRPM7 phosphorylates PLC-γ2 at residues in the linker and C2 domains (14). We expressed a PLC-γ2 mutant (PLC-γ2-T1045A), which ablates a TRPM7 phosphorylation site, in PLC-γ2-KO DT40 cells (14) and imaged these cells by live-cell TIRFM on planar lipid bilayers containing anti-IgM as surrogate antigen. These cells displayed antigen aggregation dynamics similar to that of TRPM7-KD cells (Fig. 4A): The B cell contact area was larger, and the number of antigen microclusters was increased (Fig. 4, B and C, and fig. S5). Furthermore, tracking individual microclusters revealed that microcluster diffusion was substantially reduced in PLC-γ2-T1045A cells compared to that in TRPM7-WT cells, similar to TRPM7-KD cells (Fig. 4, D and E). These findings suggest that this TRPM7 kinase target is required for BCR-antigen microcluster dynamics and centralization.

PLC-γ2 is an important phospholipase, which mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to produce inositol triphosphate and diacylglycerol (DAG). To investigate whether TRPM7 deficiency affected lipid metabolism, we stably expressed biosensors of PIP2 (25) and DAG (26) in WT, TRPM7-KO, and TRPM7-KD DT40 cells. When B cells were imaged by live-cell TIRFM, we found that PIP2 and DAG abundance increased after IgM activation, reaching a maximum at about 2 to 3 min after contact (Fig. 5A and movies S4 and S5). These kinetics are similar to those of PIP2 and DAG accumulation in activated T cells (27, 28). Although the maximal amount of PIP2 was increased in DT40 TRPM7-KO cells, less PIP2 accumulated in TRPM7-KD cells after activation (Fig. 5, A and B, and figs. S6 and S7). In contrast, there was no difference in the peak fold change of DAG in activated WT, TRPM7-KO, and TRPM7-KD cells (Fig. 5, C and D, and figs. S6 and S7), but DAG abundance after the peak was increased in TRPM7-KO and TRPM7-KD cells when compared to that in WT cells (Fig. 5D and figs. S6 and S7). These data suggest that TRPM7 modulates lipid metabolism after BCR stimulation and that TRPM7 deletion may modulate not only PIP2 hydrolysis but also PIP2 production.

Reduced TRPM7 abundance alters antigen aggregation in primary B cells

Our data have identified an important role for TRPM7 in the early events of B cell activation in a model B cell line. To test whether TRPM7 has a similar role in primary B cells, we deleted TRPM7 in murine B cells by crossing TRPM7-floxed mice with mice expressing Cre recombinase under the promoter of the pan-B cell gene CD79a-Cre (also Mb-1 or Igα). We found that B cell development in TRPM7floxed × CD79a-Cre mice was blocked at the pro-B cell stage, resulting in a complete lack of mature peripheral B cells (29). However, B cell development and the number of peripheral B cells were normal in TRPM7WTfloxed × CD79a-Cre mice heterozygous for the floxed TRPM7 allele (TRPM7+/−). The abundance of TRPM7 mRNA was reduced by about 50% in TRPM7+/− splenic B cells compared to that in WT cells (Fig. 6A). Antigen gathering as assessed by confocal microscopy showed that TRPM7+/− B cells collected 50% more antigen (Fig. 6, B and C) and spread 10% more than did WT B cells (Fig. 6, B and D) at 1.5 min after activation. Similar results were observed at 10 min after activation (Fig. 6, E to G). When these cells were stimulated with IgM, TRPM7+/− B cells exhibited stronger ERK phosphorylation after activation than did WT primary B cells (Fig. 6, F and I). The overall phenotype of primary TRPM7+/− B cells was similar to that of TRPM7-deficient DT40 B cells.

The inhibitor NS8593 directly suppresses the inward and outward ion currents of TRPM7 by binding to the magnesium-binding site (30). To assess the contribution of the ion channel function on antigen gathering mediated by TRPM7, WT DT40, and primary B cells were pretreated with NS8593, activated, and then imaged by confocal microscopy. Consistent with our observations of TRPM7-KO cells, inhibitor-treated DT40 cells acquired 1.5-fold more antigen than did dimethyl sulfoxide (DMSO)–treated control cells (fig. S8, A and B). Similarly, NS8593-treated primary B cells also accumulated more antigen than did DMSO-treated cells (fig. S8, C and D). Although NS8593 is selective for TRPM7 channels (30), the mRNAs of the more distantly related TRPM2, TRPM4, and TRPM5 channels have been detected in B cells (31). To verify the specificity of NS8593 at the concentration used in our experiments, we pretreated TRPM7-KO DT40 cells with 30 μM NS8593 before IgM activation and confocal imaging. We found that NS8593 had no effect on BCR-Ag aggregation as compared to that in vehicle-treated cells (fig. S9, A and B). However, NS8593 was originally identified in a screen of compounds developed to block Ca2+-activated K+ channels (SK channels). Because B cells express the Ca2+-activated K+ channel KCa3.1, to further verify the specificity of NS8593, we blocked SK channels and assessed antigen accumulation. Primary B cells were pretreated with 1 μM apamin, an SK channel inhibitor, and then were activated and imaged by confocal microscopy. We observed no difference in antigen accumulation between apamin-treated and vehicle-treated cells (fig. S9, C and D). Together, these findings suggest that specific inhibition of TRPM7 by NS8593 alone is sufficient to alter antigen aggregation in DT40 and primary B cells.

Fig. 4. Antigen gathering is compromised in cells expressing PLC-γ2-T1045A. (A to E) TIRFM analysis of PLC-γ2-KO cells expressing PLC-γ2-T1045A and stimulated with lipid bilayers containing fluorescently labeled anti-IgM. Images (A) and antigen microcluster tracks from individual cells (D) are representative of three independent experiments. Data on the contact area (B), the number of antigen clusters over time (C), and the diffusion coefficients of antigen microclusters (E) are means ± SEM of pooled measurements taken from at least 17 cells. Statistical significance was assessed by Mann-Whitney test and regression analysis (see fig. S5). ***p < 0.001, ****p < 0.0001. Scale bars, 5 μm.
TRPM7 is important for antigen internalization and presentation

The trafficking of BCR-antigen microclusters into endosomal compartments for processing and presentation to T cells is an essential step for full B cell activation and differentiation into antibody-producing cells. We used flow cytometry to measure the amount of surface BCR after antigen stimulation in WT DT40, TRPM7-KO, and TRPM7-KD cells. We found that WT DT40 cells internalized about 75% of the BCR after 30 min, consistent with previous observations (32). In contrast, BCR internalization was reduced in DT40 TRPM7-KO cells, with only 35% of surface BCR internalized at this time point (Fig. 7, A and B). We examined BCR internalization in TRPM7−/− primary B cells using a similar experimental approach and found no difference in BCR internalization compared to that of WT B cells (fig. S10). We also found that the kinase domain of TRPM7 was dispensable because TRPM7-KD cells internalized BCR similarly to WT cells (Fig. 7, A and B). These findings suggest that the TRPM7 channel domain, but not the kinase domain, may be important for receptor-mediated endocytosis of antigen in B cells. We cultured DT40 TRPM7-KO with 10 mM extracellular MgCl₂ and then assayed antigen internalization as described earlier but found no effect on antigen internalization in TRPM7-KO cells (fig. S11). Although these findings cannot rule out the possibility of a role for Mg²⁺ in antigen internalization, our results do indicate a specific requirement for TRPM7 in BCR-Ag internalization.

Defects in B cell antigen internalization may reduce antigen presentation to T cells. We used flow cytometry to examine the cell surface abundance of major histocompatibility complex (MHC) class II on primary murine B cells after anti-κ light chain stimulation and found about 30% less MHC class II in NS8593-treated cells than in DMSO-treated cells (Fig. 7, C and D). We also examined antigen presentation by A20 B cells that express a transgenic BCR specific for hen egg lysozyme (HEL). These cells were incubated with HEL-coated beads and either NS8593 or vehicle, and then, any non-internalized beads and inhibitor were washed out. The B cells were then cocultured overnight with the HEL-specific 2G7 T cell hybridoma, and the amount of interleukin-2 (IL-2) in the coculture medium was measured by enzyme-linked immunosorbent assay (ELISA). We found that IL-2 production was decreased about 50% when B cells were treated with NS8593 as compared to DMSO-treated cells (Fig. 7B). Together, these findings demonstrate an important role for TRPM7 in antigen internalization, presentation, and consequently, T cell activation.

DISCUSSION

Here, we showed that cellular morphology and actin organization were altered in B cells deficient in TRPM7 in both the steady state and after BCR stimulation. Although TRPM7 is implicated in the regulation of the actin cytoskeleton, its effects are contrasting depending on the cell type. In fibroblast cells, overexpression of TRPM7 elicits cell rounding and loss of adhesion (2), whereas in neuroblastoma cells, overexpression of TRPM7 increases cell spreading and cell adhesion (10). Furthermore, activation of TRPM7 by bradykinin remodels podosomes through kinase-dependent actin cytoskeleton reorganization. In vitro kinase assays TRPM7 can phosphorylate myosin IIA heavy chain, and this is important for localized relaxation of the actin cytoskeleton to allow for podosome formation (10). Conversely, small interfering RNA–mediated knockdown of TRPM7 in fibroblasts results in constricted cell morphology with exaggerated membrane extensions and further demonstrates that TRPM7 is required for the formation of lamellipodia in a wound-healing assay (15). This is consistent with our finding that TRPM7-deficient B cells lack lamellipodial extensions after stimulation with immobilized antigen. At least in fibroblasts, TRPM7 kinase activity is not required for control of actomyosin contractility, but instead, the channel acts through Rac and Cdc42 to regulate the cytoskeleton (15). TRPM7 also exerts kinase-independent regulation of the actin cytoskeleton in megakaryocytes, where deletion of TRPM7 in a murine model altered microtubule organization and content and consequently actin dynamics. However, this was not observed in a kinase-deficient knock-in model (16). In contrast, our data demonstrate that B cell morphology is disrupted in cells expressing a kinase dead mutant of
TRPM7, although the phenotype is not identical to that observed in TRPM7-KO cells. Thus, both the channel and kinase domains may play a role in regulating the actin cytoskeleton in B cells.

Our study investigated the role of the TRPM7 channel and kinase domains in regulating B cell antigen gathering, BCR signaling, and antigen internalization. We found that BCR-antigen microcluster mobility and centralization were impaired and that BCR signaling was increased in response to stimulation in TRPM7-KO and TRPM7-KD cells. This is consistent with studies demonstrating the cSMAC as the site of T cell receptor (TCR) signal attenuation (24) and that physically restricting centralization of TCR augments signaling (33). The centralization of BCR is thought to be particularly important because gathered antigen is internalized by the BCR for processing and presentation to T cells (34). The internalization of antigen for processing and presentation of peptide fragments in the context of MHC class II to CD4 T cells is a critical step in B cell activation and is required for B cells to differentiate into high-affinity antibody-producing plasma cells and memory cells (35). Both the extent of spreading and the number of microclusters formed directly affect the amount of antigen acquired, processed, and presented to T cells, and thus the amount of T cell help that the B cell receives (21). Here, we found that BCR-antigen internalization is impaired in TRPM7-deficient cells, consistent with a role for TRPM7 in the internalization of other cell surface proteins, including Fas receptor (36) and Toll-like receptor 4 (13).

Although the mechanism for TRPM7-mediated regulation of receptor endocytosis has not been identified, our finding is consistent with a key role for actin in antigen internalization; both actin depolymerization (37, 38) and altered Syk-dependent reorganization of the actin cytoskeleton (39) decrease the efficiency of BCR-antigen internalization and convergence with MHC class II–containing endosomal compartments. Moreover, trafficking of internalized BCR to MHC-containing lysosomes involves the motor protein myosin II (40). If TRPM7 kinase phosphorylates myosin IIA heavy chain (10), although TRPM7 kinase dead cells internalize BCR with kinetics similar to WT cells, then the intracellular trafficking and convergence with MHC II–containing lysosomes may be compromised.

We interrogated the kinase versus channel function of TRPM7 in B cell activation and found that the kinase domain was necessary for antigen gathering during B cell contraction but was dispensable for antigen internalization. The kinase domain of TRPM7 phosphorylates Thr1045 in the linker region of PLC-γ2 (14), a key BCR signaling molecule involved in actin reorganization and BCR microcluster formation (19). In PLC-γ2-KO DT40 B cells that expressed the T1045A mutant of PLC-γ2, we found that BCR-antigen microcluster dynamics and aggregation were defective, similar to those in TRPM7-KD cells. Although it is currently not well understood how phosphorylation of this residue affects PLC-γ2 activity, the C2 domain helps to stabilize Ca2+-dependent binding to the adaptor protein SLP-65 (BLNK) for sustained PLC-γ2 signaling (41). TRPM7-mediated phosphorylation of residues within the C2 domain (14) may reduce PLC-γ2 activity or association with SLP-65. Thus, in the absence of TRPM7 kinase activity, PLC-γ2 activity may be sustained, which would result in ongoing hydrolysis of PIP2 into DAG.

We investigated the activation of lipid second messenger signaling in TRPM7-KO and TRPM7-KD cells using green fluorescent protein (GFP)–tagged biosensors of PIP2 and DAG. We found that the amount of DAG was increased at later times after activation in both TRPM7-KO and TRPM7-KD cells compared to that in WT cells. Because DAG feeds into the RasGRP-MAPK (mitogen-activated protein kinase) pathway, this sustained DAG activity was consistent
proteins, such as cofilin and gelsolin (actin-related proteins 2/3, but also impair the activity of actin-nucleating proteins, such as Wiskott-Aldrich syndrome protein and influencing the activity of several proteins involved in actin remodeling and is important for BCR microcluster mobility (3)).

In addition to playing a key role in the regulation of the actin cytoskeleton, PIP2 is also involved in clathrin-mediated endocytosis (42). Large amounts of PIP2 not only increase the activity of actin-nucleating proteins, such as Wiskott-Aldrich syndrome protein and actin-related proteins 2/3, but also impair the activity of actin-severing proteins, such as cofilin and gelsolin (42). Conversely, low PIP2 abundance blocks actin polymerization. Thus, the finding of dysregulation of PIP2 activity in TRPM7-KO and TRPM7-KD cells is broadly consistent with our observation that these cells also exhibit altered cellular morphology and actin organization. We noted that actin reorganization after BCR stimulation was more affected in TRPM7-KD cells than in TRPM7-KO cells. Although we found that the overall amount of PIP2 was reduced in TRPM7-KD cells, we did find ongoing localized PIP2 in the periphery of the cell contact. This may disrupt the dynamic reorganization of actin, perhaps by inhibiting actin-severing proteins, such as cofilin, which is activated upon BCR stimulation and is important for BCR microcluster mobility (45).

In addition to playing a key role in the regulation of the actin cytoskeleton, PIP2 is also involved in clathrin-mediated endocytosis (46). PIP2 is a main binding partner for several endocytic proteins and regulates the initiation and stabilization of clathrin-coated pits (46). However, increased PIP2 abundance is associated with non-productive endocytosis, suggesting that the turnover of PIP2 is necessary for endocytosis (47). It has been suggested that the defect in PIP2 turnover may impair actin disassembly necessary for endocytosis or cause failure of individual endocytic events, such as vesicle scission (47). Thus, the finding of defective antigen internalization in TRPM7-KO cells may be reflective of nonproductive endocytosis due to increased PIP2 abundance. Moreover, this may explain why antigen internalization is not impaired in TRPM7-KD cells because PIP2 abundance is not increased in these cells.

We also investigated a role for the channel domain of TRPM7 in our observed phenotype. TRPM7 is critical for the maintenance of cellular Mg2+ homeostasis, and growth defects in TRPM7-deficient DT40 cells can be rescued by supplementation with extracellular Mg2+ (3). In contrast, we found that supplementation with extracellular Mg2+ had no effect on the antigen gathering or internalization defect in TRPM7-KO cells. Although supplementation with 10 mM Mg2+ was sufficient to rescue the growth defect in TRPM7-KO cells, we cannot be sure that Mg2+ uptake by other magnesium ion channels is sufficient to restore physiological amounts of intracellular Mg2+. However, supplementation with extracellular Mg2+ reduced ERK phosphorylation in cells lacking TRPM7 after BCR stimulation. Although the reason for this is not clear, divalent cations are required for the activity of DAG kinase ζ (DGKζ) (48), one of the principal isoforms expressed in immune cells (49). DGKζ inhibits DAG signaling by phosphorylating DAG to produce phosphatidic acid. Deletion of DGKζ in primary B cells results in increased ERK signaling in mature follicular B cells (50). Thus, DGKζ activity may be attenuated in TRPM7-KO cells that lack sufficient Mg2+. However, it was somewhat surprising that ERK signaling was attenuated by Mg2+ in TRPM7-KD cells because the channel is still operational in these cells. There may be functional coupling between the kinase and channel domains (3), which may affect the amount of intracellular Mg2+ available for binding to and activating DGKζ. Although our study points to a role for TRPM7 in the regulation of lipid second messenger signaling, future studies further defining the molecular targets downstream of the Mg2+ transport function and kinase activity of TRPM7 are warranted and may yield new targets for therapeutic treatment of disease.

Fig. 7. TRPM7 is important for antigen internalization and presentation. (A and B) Flow cytometry analysis of surface BCR expression on WT, TRPM7-KO, and TRPM7-KD DT40 B cells after IgM stimulation. Histograms (A) are representative of three independent experiments. Quantified fold change in the mean fluorescence intensity (MFI) (B) is means ± SEM. (C and D) Flow cytometry analysis of surface MHC class II expression on primary murine B cells after anti-μ stimulation with vehicle or NS8593 (channel inhibitor). Histograms (C) are representative of three independent experiments. Quantified MFI (D) data are means ± SEM. (E) A20 B cells were treated with vehicle or NS8593 and incubated with beads coated with HEL. Cells were mixed with HEL-specific 2G7 T cell hybridoma, and antigen presentation was assessed by ELISA measurement of IL-2 secretion 18 hours after stimulation. Fold change IL-2 concentration data representative of three independent experiments are means ± SEM. Statistical significance was assessed by one-way ANOVA with Tukey’s post hoc test or Mann-Whitney (E). *P < 0.05.

with our finding that phosphorylation of ERK is increased in TRPM7-KO and TRPM7-KD mutant cells. We found that the amount of PIP2 was increased in TRPM7-KO cells but was decreased in TRPM7-KD cells. This implies that PIP2 production may be differentially affected in TRPM7-KO cells versus TRPM7-KD cells, although the underlying mechanism for this difference requires further investigation. PIP2 regulates the actin cytoskeleton by binding to and influencing the activity of several proteins involved in actin remodeling (42–44). Large amounts of PIP2 not only increase the activity of actin-nucleating proteins, such as Wiskott-Aldrich syndrome protein and actin-related proteins 2/3, but also impair the activity of actin-severing proteins, such as cofilin and gelsolin (42). Conversely, low PIP2 abundance blocks actin polymerization. Thus, the finding of dysregulation of PIP2 activity in TRPM7-KO and TRPM7-KD cells is broadly consistent with our observation that these cells also exhibit altered cellular morphology and actin organization. We noted that actin reorganization after BCR stimulation was more affected in TRPM7-KD cells than in TRPM7-KO cells. Although we found that the overall amount of PIP2 was reduced in TRPM7-KD cells, we did find ongoing localized PIP2 in the periphery of the cell contact. This may disrupt the dynamic reorganization of actin, perhaps by inhibiting actin-severing proteins, such as cofilin, which is activated upon BCR stimulation and is important for BCR microcluster mobility (45).

MATERIALS AND METHODS
Cell preparation and culture
WT, TRPM7-KO (3), and PLC-γ2-KO cells stably expressing PLC-γ2-T1045A (14) DT40 B cells were maintained at 39.5°C with 5% CO2 in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 1% chicken serum, penicillin and streptomycin (100 U/ml) (all from Gibco), 50 μM 2-mercaptoethanol (Amresco), and 10 mM MgCl2. DT40 B cells were transferred into medium containing 2 mM MgCl2, 18 hours before experiments were performed. Expression of TRPM7-K1648R (TRPM7-KD) or human TRPM7 (TRPM7-hWT) (3) in TRPM7-KO cells was induced with doxycycline (10 μg/ml) before analysis. A20

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B cells expressing HEL-specific BCRs (D1.3) and the 2G7 T cell hybridoma were maintained at 37°C with 5% CO2 in RPMI 1640 containing 10% heat-inactivated FBS, penicillin and streptomycin (100 U/ml), and 50 μg 2-mercaptoethanol. C57BL/6 mice were obtained from Charles River Laboratories. 129/SvEvTac TRPM7flox/flox mice were purchased from the Jackson Laboratory and were crossed with C57BL/6 CD79a-cre mice, provided by M. Reth (AT). TRPM7flox/+ CD79a-cre/+ (TRPM7flox/wt) and TRPM7flox/flox (WT) were maintained on a mixed 129/SvEvTac and C57BL/6 background. Primary murine splenic B cells were isolated by negative selection according to the manufacturer’s protocol (Miltenyi Biotec). All experiments were approved by the Local Animal Care Committee at the University of Toronto, Scarborough.

**Generation of biosensor DT40 B cell lines**
Mammalian expression vectors that encode the Cys1 domain of protein kinase Cy (PKCy) fused to GFP and the pleckstrin homology (PH) domain of PLCδ2 fused to GFP were generated previously (25, 26). WT, TRPM7-KO, and TRPM7-KD DT40 B cells were electroporated with 10 μg of linearized DNA using a Gene Pulser Xcell (Bio-Rad) at 570 V, 25 μF. Single clones were selected with hygromycin (5 mg/ml).

**Scanning electron microscopy**
Cells on glass coverslips were fixed with 2% glutaraldehyde for 1 hour at room temperature, followed by post-fixation in 1% OsO4 for 2 hours. Fixed cells were then dehydrated in 50, 70, 95, and 100% ethanol, critical point–dried with liquid CO2, and sputter-coated with gold. Images were acquired with a scanning electron microscope (Hitachi S350). Surface protrusions adjacent to the coverslip were manually quantified regardless of length by drawing a line along the length of the protrusion in Velocity software (Improvision).

**Confocal microscopy**
All staining was performed in FCS2 imaging chambers or on glass coverslips coated with anti-chicken IgM (5 μg/ml; clone M4, provided by F. Batista) in phosphate-buffered saline (PBS) overnight at 4°C. Cells were settled on coverslips for 10 min at 37°C, then fixed in prewarmed 4% paraformaldehyde for 15 min at 37°C, permeabilized with PBS containing 0.1% Triton X-100 for 5 min, and blocked in 1% bovine serum albumin for 30 min at room temperature. Cells were stained for F-actin with Alexa Fluor 488–conjugated phalloidin (Invitrogen). Confocal images were acquired with a spinning disc confocal microscope (Quorum Technologies) consisting of an inverted fluorescence microscope (DMi6000B, Leica) equipped with a 63×/1.4 numerical aperture (NA) oil immersion objective and an ORCA-R2 camera.

**Total internal reflection fluorescence microscopy**
Artificial planar lipid bilayers containing anti-IgM (clone M1) or anti-κ (clone HB58, provided by F. Batista) as a surrogate antigen were prepared in FCS2 chambers (Bioptechs) by liposome spreading, as previously described (19). 1,2-dioleoyl-sn-glycero-3-phosphocholine liposomes (Avanti Polar Lipids Inc.) were mixed with either 1.0 × 10⁻³ or 2.5 × 10⁻⁴% biotinylated liposomes for the preparation of lipid bilayers for DT40 and primary B cell experiments, respectively. Alexa Fluor 633–conjugated streptavidin (Invitrogen) was incorporated into the lipid bilayers and used to tether biotinylated anti-mouse κ light chain (clone HB-58) or anti-chicken IgM (clone M1). Assays were performed in chamber buffer [PBS, 0.5% FBS, 2 mM Mg²⁺, 0.5 mM Ca²⁺, 1 g/liter]. TIRFM, IRM, and differential interference contrast images were all acquired using a TIRF microscope (Quorum Technologies) consisting of an inverted fluorescence microscope (DMi6000B, Leica), HCX PL APO 100×/1.47 NA oil immersion objective, and Evolve Delta EMCCD camera (Photometrics).

**Western blotting**
DT40 B cells (WT, TRPM7-KO, and TRPM7-KD) or primary B cells (WT or TRPM7flox/wt) were equilibrated in RPMI 1640 at 37°C for 10 min and plated onto immobilized stimulatory anti-IgM (clone M1, provided by F. Batista) or polyclonal goat anti-mouse IgM F(ab)′2 (Jackson ImmunoResearch) for the times indicated in the figure legends at 37°C. Cells were lysed in 2× Laemmli sample buffer, resolved by 12% SDS–polyacrylamide gel electrophoresis, and analyzed by Western blotting with antibodies against phospho-p44 and phospho-p42 MAPK (ERK1 and ERK2) or total ERK1/2 (Cell Signaling Technology) after stripping the blots with mild stripping buffer [0.2 M glycine, 3.5 mM SDS, 0.01% Tween 20 (pH 2.2)] for 13 min. Densitometric analysis of Western blots was performed with Image software. The amount of pERK was normalized to loading control and then expressed as a fold change relative to the 0-min time point.

**Quantitative reverse transcription polymerase chain reaction**
Splenoocytes were isolated from WT and TRPM7flox/wt mice, and B cells were purified with a mouse B cell isolation kit (STEMCELL Technologies). RNA from 5 × 10⁶ B cells was isolated with the RNeasy Mini Kit (Qiagen). Synthesis of complementary DNA (cDNA) was completed with the qScript cDNA Synthesis Kit (Quanta BioSciences) in the T100 ThermoCycler (Bio-Rad). One-fifth of the cDNA first-strand reaction was used to perform qPCR with Power SYBR Green Mastermix (Applied Biosystems). Primer sets used for the reactions were synthesized by Sigma-Aldrich using previously published sequences (52, 53): TRPM7, 5′-TTTTGGTGTTCCAGAAGACG-3′ (sense) and 5′-ACCAAAGTTCGACACAG-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TCAA-CAGACACTCCACTCT-3′ (sense) and 5′-ACCCGTCTTGGTAGCCTGA-3′ (antisense). The qPCR reactions were performed in the CFX Connect Real-Time PCR Detection System (Bio-Rad). The fold change in mRNA abundance between WT and TRPM7flox/wt B cells was quantified by the ΔΔCt (cycle threshold) method after normalization to the abundance of GAPDH mRNA. Student’s t test (Prism version 6.01, GraphPad Software) was used to determine statistical significance.

**Flow cytometric assay for BCR internalization**
DT40 cells were treated with anti-chicken IgM (10 μg/ml; clone M4) in PBS on ice for 20 min before washing unbound antibodies three times with 5 ml of ice-cold PBS. Cells were warmed to 37°C for the times indicated in the figure legends. The internalization process was then stopped by the addition of 1 ml of ice-cold PBS containing 3% FBS, 0.2% NaN₃. Surface BCR was stained with primary anti-chicken IgM (clone M1) antibody, followed by Alexa Fluor 488–conjugated goat anti-mouse secondary antibody in FACs buffer. Purified primary B cells were equilibrated in RPMI 1640 at 37°C for 10 min and then stimulated with anti-κ (5 μg/ml; clone HB58) at 37°C. The internalization process was stopped at the times indicated in the figure legends with ice-cold PBS containing 3% FBS, 0.2% NaN₃. Surface IgD was

stained with allophycocyanin-conjugated anti-mouse IgD (clone 11-26c.2a, BioLegend). Analysis was performed by flow cytometry (Fortessa, BD Biosciences) and FlowJo software (Treestar).

**MHC class II up-regulation assay**

Murine splenocytes (4 x 10⁵ cells) were incubated in complete media containing B cell activating factor (50 µg/ml), with or without 30 µM NS8593 (Sigma-Aldrich) for 10 min at 37°C. Cells were then stimulated with anti-κ (clone HB58, provided by F. Batista)–coated polystyrene beads (110 nm, Bangs Laboratories), incubated for 18 hours at 37°C, followed by immunostaining for B220, CD19, and MHC class II, and analyzed by flow cytometry.

**Presentation assay**

A20 D1.3 B cells (1 x 10⁵ cells) were treated with 30 µg/ml), with or without 30 µM NS8593 (channel inhibitor) for 10 min at 37°C before the addition of HEL (Sigma-Aldrich)–coated polystyrene beads (110 nm, Bangs Laboratories). Cells were allowed to internalize the beads for 1 hour before being washed of inhibitor. B cells were then added to the 2G7 T cell hybridoma at a 1:1 ratio. B and T cells were cocultured at 37°C and 5% CO₂ for 18 hours. The cells were then freeze-thawed to release IL-2 before determining its concentration by ELISA (BD OptEIA).

**Image processing and data analysis**

Quantification of antigen microcluster tracking, the contact area of each B cell with planar lipid bilayers, and the number of microclusters was performed with MATLAB software (version R2014a, MathWorks) (23, 54). Microclusters were defined as an enrichment of at least twice the fluorescence intensity of the background. The fluorescence intensities of antigen, F-actin, and shape factor were quantified with the thresholding feature in Volocity for automatic detection (version 5.0, PerkinElmer). The relative enrichment of microclusters was determined using the plot intensities along line function in Volocity. Antigen was pseudocolored using the rainbow lookup table provided by Volocity. PLCγ1–PH–GFP and PKCγ1–GFP were pseudocolored using the fire lookup table provided in ImageJ (National Institutes of Health). Statistical significance was assessed with Prism (version 6.01, GraphPad Software) or R using Mann-Whitney, ANOVA, or regression analysis as indicated in the figure legends.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Statistical analysis of the spreading and microcluster accumulation in WT and TRPM7-KO DT40 B cells.

Fig. S2. Expression of TRPM7 in TRPM7-KO cells restores B cell antigen gathering.

Fig. S3. Statistical analysis of the spreading and microcluster accumulation in WT and TRPM7-KD DT40 B cells.

Fig. S4. Supplementation with extracellular Mg²⁺ is not sufficient to rescue the BCR internalization defect in TRPM7-KO cells.

Movie S1. B cell spreading and the contraction response are impaired in TRPM7-KO DT40 cells.

Movie S2. TRPM7 kinase activity is important for cell contraction.

Movie S3. Antigen gathering is compromised in cells expressing PLCγ1–GFP and Vav and Vav channel activities.

Movie S4. The amount of PIP2 at the cell-bilayer interface is altered in TRPM7-KO and TRPM7-KD cells upon activation.

Movie S5. The amount of DAG at the cell-bilayer interface is altered in TRPM7-KO and TRPM7-KD cells upon activation.

**REFERENCES AND NOTES**


Ion channel activity controls antigen uptake

The TRPM7 magnesium channel is one of only two ion channels that also contain a kinase domain. This dual ion channel–kinase can phosphorylate nonmuscle myosin II A heavy chain and control cytoskeletal rearrangements. Using TIRF microscopy, Krishnamoorthy et al. found that expression of TRPM7 in B cells controlled actin dynamics and prevented antigen internalization. In activated B cells lacking TRPM7 or TRPM7 kinase activity, more antigen accumulated on the cell surface and activated stronger B cell receptor-dependent signaling. An inhibitor of TRPM7 ion channel activity reduced antigen presentation to T cells. These data identify a previously uncharacterized role for TRPM7 in B cell antigen uptake and presentation.