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Class I myosins in B-cell physiology: functions in spreading, immune synapses, motility, and vesicular traffic

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Summary: Myosins comprise a family of motor proteins whose role in muscle contraction and motility in a large range of eukaryotic cells has been widely studied. Although these proteins have been characterized extensively and much is known about their function in different cellular compartments, little is known about these molecules in hematopoietic cells. Myosins expressed by cells from the immune response are involved in maintaining plasma membrane tension, moving and secreting vesicles, endo- and exocytotic processes, and promoting the adhesion and motility of cells. Herein, we summarize our current understanding of class I myosins in B cells, with an emphasis on the emerging roles of these molecular motors in immune functions.

Keywords: *B lymphocyte, cytoskeleton, myosin I*

Introduction

Lymphocytes are cells with an incredible array of cellular functions. Their most important job is the recognition of self/non-self antigens through highly specialized receptors. During the last 40 years, antigen receptors, costimulatory molecules, adhesion receptors, chemokine, and homing receptors, etc. have been described using many different strategies; however, the role of the cytoskeleton and the connection between these surface receptors and the proteins inside the cells have been poorly analyzed. The varied functions of the cytoskeleton include determining shape and motility, controlling the mechanical resistance of the membrane, and establishing pathways for the transportation of internal components from the sites of synthesis to the places of action.

Our group initially became interested in the cytoskeleton after reproducing the observation initially made by Cambier and Lehmann (1), who reported that activated B lymphocytes are able to spread over a surface precoated with anti-major histocompatibility complex class II (MHC-II) monoclonal antibodies (mAbs). At that time, we worked on

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the characterization of a series of monoclonal antibodies, recognizing surface molecules on resting and activated B lymphocytes. By using immobilized antibodies on plastic plates, we found that not only were anti-MHC-II mAbs able to induce spreading, but other molecules were also efficient in inducing similar phenomena (2). The first molecule we found that induced spreading was an antibody recognizing CD44, an activation molecule present in both T and B cells. We analyzed several antibodies recognizing different epitopes of CD44, and all of them were equally efficient in inducing spreading (3). As was reported by Cambier (1), spreading is only noted in activated B lymphocytes, because resting cells do not change their morphology when cultured under similar circumstances. The morphology of cells seen under the microscope showed the generation of long and pleomorphic appendages resembling the morphology of neurons (*Fig. 1A*); staining with phalloidin revealed an amazing array of fine dendrites all along the body of the cell (*Fig. 1B–D*). There was no obvious polarity, since dendrites could appear from one site or from multiple sites of the cell. Moreover, those dendrites were not static structures; they changed with time. The production of these structures was dependent on temperature and energy, because reducing incubation temperature decreased and prevented their formation, and the addition of low amounts of sodium azide also had catastrophic consequences: in minutes, all cells which had been spreading became rounded (J.L. Maravillas-Montero, O. López-Ortega, G. Patiño-López and L. Santos-Argumedo, submitted manuscript).

Staining with phalloidin showed discrete and organized microfilaments of actin, organized in the dendrites and the tiny fine microvilli. All new appendages showed an accumulation of F-actin, later becoming stabilized by tubulin. Not surprisingly, dendrite formation was inhibited by cytochalasin D, colchicine, and nocodazole. Dendrites also showed reorganization of vimentin intermediate filaments and accumulation of the ezrin/radixin/moesin (ERM) family of proteins (4).

As mentioned above, anti-MHC-II and anti-CD44 antibodies equally induced spreading; interestingly, some other molecules showed a similar property in inducing dendrite formation of activated B cells. Antibodies against intercellular adhesion molecule-1 (ICAM-1), leukocyte function-associated antigen-1 (LFA-1), and CD45R, among others, induced spreading. However, not all surface molecules were equally able to induce this phenomenon (5).

Because activation is a prerequisite for the cells to spread, we analyzed different stimuli. Stimulation by several receptors such as the B-cell receptor (BCR), CD40, CD38, and Toll-like receptor-4 equally prepared the cells for spreading. IL-4 was one of the most important stimuli. On its own, it induced a migratory morphology in most B cells, but its combination with any of the stimuli mentioned above induced the most dramatic forms of spreading (2) (*Fig. 2*). Studies from the Severinson laboratory (6) have also linked motility response and spreading to IL-4 stimulation, with the participation of signal transducer and activator

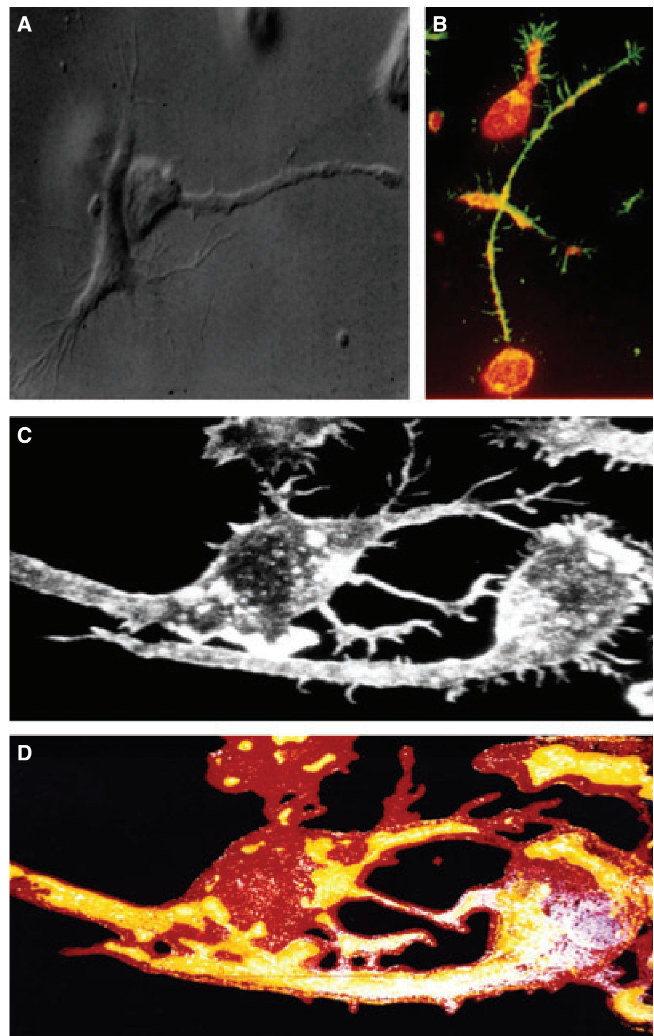

Fig. 1. **Actin cytoskeleton plasticity observed during B-cell spreading.** Pre-activated mouse B lymphocytes were set over plates coated with antibodies, such as anti-CD44 or anti-MHC-II, to induce spreading. During this process, the cells extend multiple membrane extensions easily detected by bright field microscopy (A) that resemble neuronal projections. (B). Spread B lymphocytes stained with FITC-phalloidin (green) to denote microfilaments and DNAse (red) to label the nuclei. (C) Magnification of a spread B cell stained with the same dyes displaying both long and thicker dendrite-like extensions and small thin filopodia; the digital representation of the same image is presented in (D), showing the DNAse stain in red and the F-actin stain in yellow.

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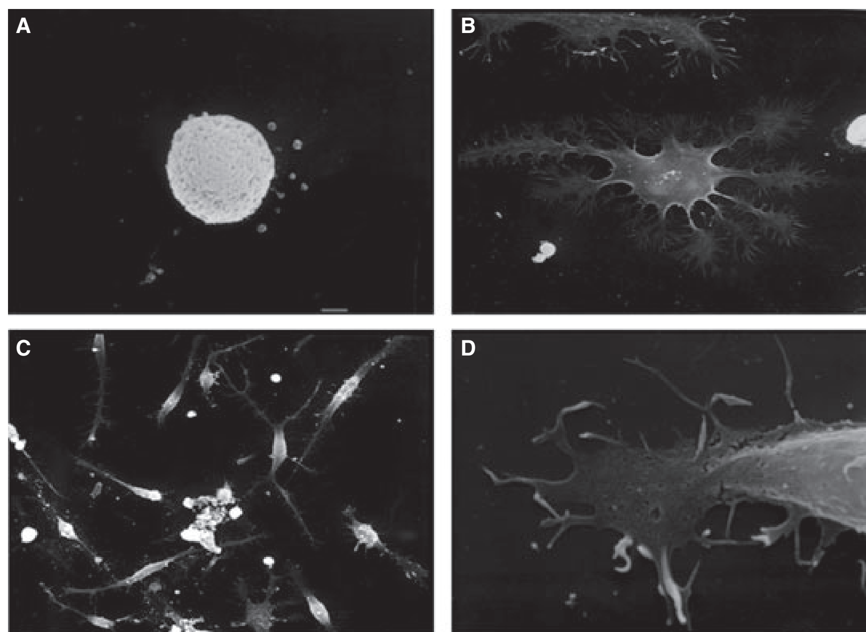

Fig. 2. Dramatic shape changes in B lymphocytes during spreading. Scanning electron microscopy images showing B lymphocytes at resting conditions (A), or after inducing the spreading with plate-bound anti-CD44 or anti-MHC-II (B, C, D). It is clear that the initial and characteristic rounded shape of the cells (A) is dramatically altered after the cytoskeletal reorganization observed during spreading. The spread cells display multiple and complex webs of plasma membrane extensions with different lengths and extension areas.

of transcription 6 as a transduction pathway. However, little is known about how these receptors are linked to the reorganization of the different elements of the cytoskeleton and the signaling pathways involved in such phenomena. Among other signaling molecules, Calcium and protein kinase C are essential, because pharmacological interference prevents or reverts both motility and spreading (2). For obvious reasons, phospholipase C-γ (PLC-γ) then is also necessary to generate the signaling molecules described above (5). Linker molecules such as the ezrin–radixin–moesin (ERM) family of proteins are activated both by interaction with phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] in the membrane and by phosphorylation; they are known to interact with CD44 to form a bridge with actin microfilaments (7–9). During spreading, ERM proteins are equally reorganized and bound preferentially in places where CD44 is located. These interactions are not been fully explored for their relevance during spreading, but they are abundant in the microvilli and probably maintain surface molecules such as CD44, ICAM-1, and LFA-1 in these tiny structures (10, 11).

These membrane protrusions have an important protein content, comprising adhesion molecules, endosomal trafficking molecules, and cytoskeleton-associated proteins (like myosins), among others. The proteins associated to the actin cytoskeleton are very important, because these proteins are the bridge between the plasma membrane and the actin cytoskeleton. Myosin deficiency affects the microvillar architecture; for example, deficiency of myosin-Ia (Myo1a) produces herniations in the apical region in a subset of enterocytes (12).

Myosins represent a large family of motor proteins with diverse functions in many cells and tissues. Muscular myosin is the best studied and most characterized protein; however, non-muscular myosins are, in fact, widely distributed and have many different and diverse functions. The role of these motor proteins in the physiology of lymphocytes is relatively unknown and, recently, has been a matter for analysis in several laboratories. Reports published recently focus on understanding the role of myosins II and V, but the expression and function of other myosins is meager or frankly missing.

Reports about the role of motor proteins in the motility of lymphocytes led us to identify these molecules in B cells. Although both information and reagents were scarce, we used the tools available for determining the presence and participation of myosins in B-cell spreading. First, we used a pharmacologic approach, using 2,3-butanedione monoxime (a rather non-specific myosin inhibitor); we found a dose-dependent inhibition of spreading (5). Next, by using different cross-reactive antibodies, we searched for myosin. The best results obtained were with an antibody against myosin from *Acanthamoeba castellanii* (a kind gift from Dr.

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Isaura Meza) that recognizes myosin-1 (Myo1) (J.L. Maravillas-Montero, O. López-Ortega, G. Patiño-López and L. Santos-Argumedo, submitted manuscript). By using the peptide sequence recognized by that antibody as bait, we found similarity with myosin-Ic (J.L. Maravillas-Montero, O. López-Ortega, G. Patiño-López and L. Santos-Argumedo, submitted manuscript). With the kind collaboration of Dr. Peter Gillespie from The Oregon Health & Science University in the USA, we used specific reagents against mouse myosin 1c (Myo1c) that allowed us to corroborate our initial observations, indicating differential localization of Myo1c during B-cell spreading (5).

Regardless of the exact nature of spreading and its role in B-cell physiology, we used this assay for initial screening because it is simple to perform, highly reproducible, and provides a very useful tool for analyzing interactions between surface receptors and cytoskeleton. Some authors question the physiology of this assay, contrasting it with the equally artificial analysis via synthetic planar membranes. However, we believe both assays can provide useful initial data about the connection between surface molecules and the cytoskeleton during the recognition of antigens and the recruitment of costimulation. Finally, activated B cells are not sessile, passive components of the immune response, but active participants that use the help they need for further proliferation and differentiation.

Myosin studies in B cells

Molecular motors represent nano-engines that generate directional movement using chemical energy. Historically, since the muscle myosins were the first to be characterized, they receive the designation of 'conventional', whereas the rest of the myosins expressed elsewhere were called 'unconventional'. The discovery and extensive research of unconventional myosins exposed the importance of these motors in several cellular processes, such as transport of organelles, transport of protein-RNA complexes, cytokinesis, control of membrane dynamics, mechanotransduction of signals in the inner ear hair cells and in stress fibers, and even gene transcription regulation (13–15).

These motors usually are composed of one or two heavy chains plus a variable number of associated light chains (15). Thus far, close to 40 different myosin classes have been identified, classified by their amino acid sequence homology and the presence of conserved domains in the heavy chain (16). The basic molecular architecture of a myosin heavy chain features an N-terminal catalytic motor domain that both hydrolyses ATP and binds to F-actin, a neck domain that binds the light chains and acts as a lever arm, and a C-terminal tail domain, which is responsible for the specific function of the given myosin as it allows the association with other molecules by the presence of interaction domains, such as Src-homology 3 domain (SH3), PH, rhoGAP, zinc finger, etc. (15).

The presence of unconventional myosins in hematopoietic cells has typically been associated with cell motility (17–20); however, now, these versatile molecular motors have been identified as regulators of totally different cellular functions. In the context of B cells, the main reports about the participation of these kinds of molecules in biological processes are commonly related with the antigen uptake ability (21–23).

Because the activation of B lymphocytes depends primarily on the recognition of specific antigens by the BCR, the processes involved in antigen recognition have an important role. By using lipid membranes or antigen-loaded dendritic cells (DCs), it has been shown that the class II unconventional myosin IIA (MyH9 or NMMH-IIA) is one of the main regulators of antigen uptake, since it exerts the mechanical forces necessary for the B cell to pinch antigen clusters off the presenting membranes. Thus, MyH9 is required for membrane-tethered antigen recognition and further internalization, a prerequisite for subsequent antigen presentation to T cells. Although there is some evidence for the role of MyH9 in antigen presentation, the possibility that other myosins could be participating in these processes is not excluded (22).

Class I myosins are of particular interest, since some are reported to be highly expressed in lymphocytes; however, little information exists about the specific roles of class I myosins in these cells. Myosins in this particular class have a unique heavy chain with a characteristic tail domain (TH1) that promotes direct binding to phosphoinositides in the plasma membrane (24, 25).

Vertebrate class I myosins are formed by just one heavy chain of 110–140 kDa (26, 27) and, unlike conventional myosin II present in muscular and non-muscular cells, do not form filaments (*Fig.* 3). In this myosin heavy chain, we can distinguish the head (or motor), the neck, and the tail domains (*Fig.* 3). The motor or head domain contains the ATP-binding site and an actin-binding site, then, it is the region responsible to generate movement.

The motor domain is followed by the neck domain, a region that includes one or more IQ motifs. These motifs are composed by near to 30 amino acids with the consensus sequence I-Q-X-X-X-R-G-X-X-X-R (28). The main function

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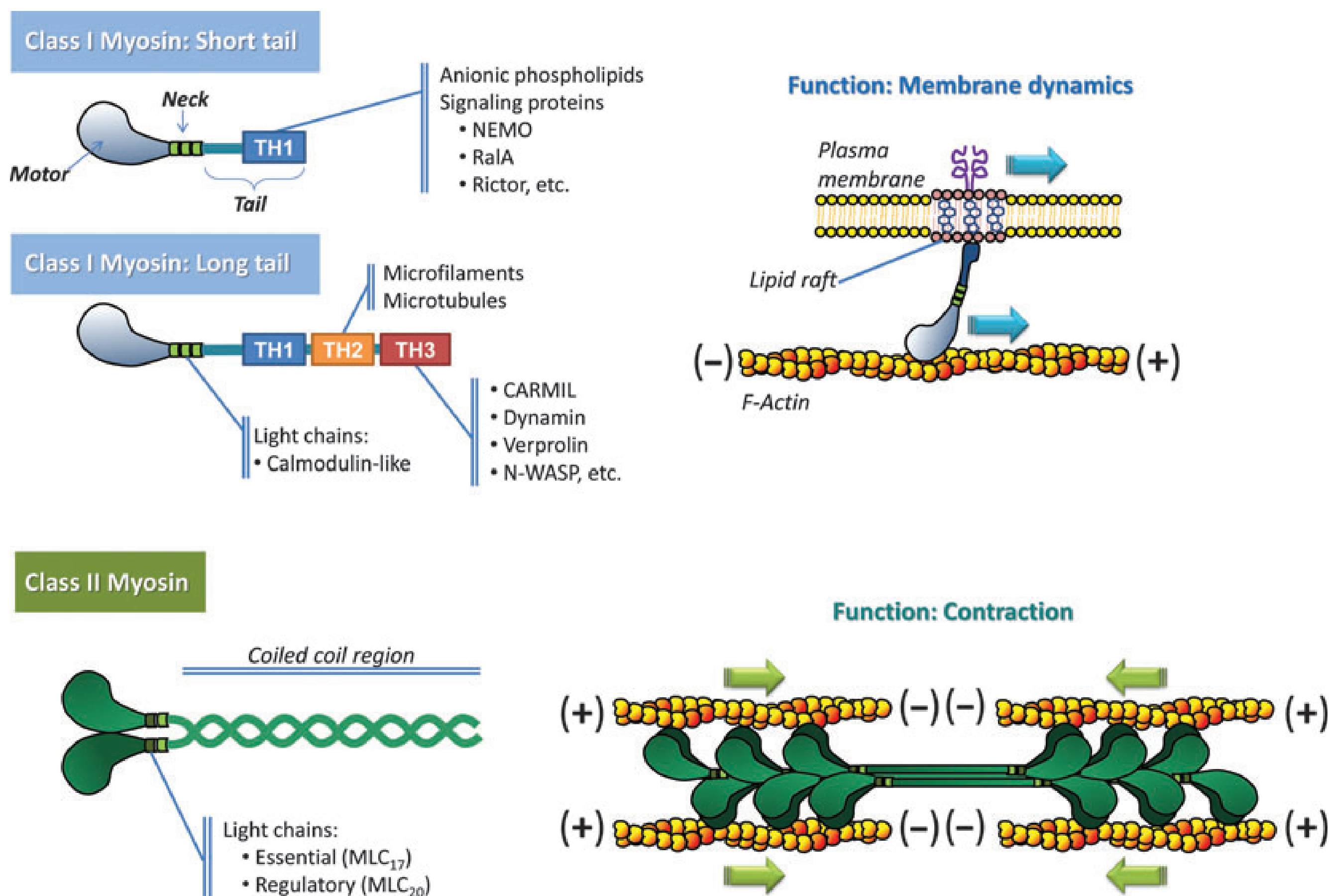

Fig. 3. Class I versus class II myosins: differences in structure and functionality. Class I myosins differ substantially to the class II myosins. They are formed by the motor, neck, and tail domains. The latter ones present differences between the short-tailed and the long-tailed class I myosins. The short-tailed myosins include only the TH1 region that allows membrane association. The long-tailed sub-class presents the additional TH2 and TH3 motifs, which are sequences that support the binding of several binding partners, like the ones displayed here. Functionally, the class I myosins are related with membrane modification/stabilization processes while the class II myosins are well known for exert contraction forces 'pulling' microfilaments at the muscle and non-muscle cells.

of this neck region is the binding of myosin calmodulin light chains, which regulates the activity of the class I myosins (29).

The C-terminal region of the myosin heavy chain is known as the tail domain. The tail domain is functionally divided into three tail homology regions (TH1, TH2, and TH3) (30). The TH1 region is enriched in basic amino acid residues, a feature that allows its binding to membrane anionic phospholipids such as phosphoinositides (31, 32). The TH2 region is rich in glycine, proline, and alanine/glutamine residues and contains an ATP-insensitive actin-binding site (33). It has also been reported that the TH2 region allows microtubule binding (34), making this region an attractive candidate to link both microtubule and microfilament cytoskeleton webs. The TH3 domain represents a SH3 (35), which is a protein-protein interaction region that allows the binding of several interacting partners (*Fig.* 3).

Class I myosin heavy chains that present all three TH regions are known as 'long-tailed', whereas those containing only the TH1 region are named as 'short-tailed' (*Fig.* 3). In the human and the mouse, eight different genes encode the eight heavy chains of class I myosins. Myosin 1a (Myo1a), myosin 1b (Myo1b), Myo1c, myosin 1d (Myo1d), myosin 1g (Myo1g), and myosin 1h (Myo1h) represent the short-tailed class I myosins whereas myosin 1e (Myo1e) and myosin 1f (Myo1f) are long-tailed (36).

The microarray data depicted in *Table 1* (29, 37) shows that mouse splenic B lymphocytes express Myo1c, Myo1e, and Myo1g as their main class I myosins. An interesting observation arises from the fact that the expression levels of these three molecules changes according to the maturation stage of B cells; there are no reports that any of these myosins could be related with B-cell ontogeny, but the possibility is attractive.

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***Table 1.* Class I myosin expression in immune cell populations**

Population/Subpopulation	Myo1a	Myo1b	Myo1c	Myo1d	Myo1e	Myo1f	Myo1g	Myo1h
Long-term repopulating hematopoietic stem cells	38	27	347	269	94	417	274	65
Short-term repopulating hematopoietic stem cells	36	28	488	160	63	625	442	73
ProB cells	45	46	909	76	63	156	389	46
PreB cells	38	29	1931	64	126	52	575	40
Spleen follicular B cells	34	30	1560	88	2146	234	920	49
Spleen germinal center B cells	44	31	1256	84	2509	89	991	46
Spleen marginal zone B cells	39	36	1187	74	1903	182	726	48
Peritoneal B-1a cells	40	30	1590	255	2604	104	1204	82
Thymus CD8+ dendritic cells	42	31	535	42	95	1459	1420	128
Spleen CD4+ dendritic cells	43	251	395	75	362	2162	1106	184
Spleen CD8+ dendritic cells	48	33	442	70	70	1224	1000	105
Spleen CD8+ plasmacytoid dendritic cells	39	27	690	52	63	505	487	275
Spleen CD8− plasmacytoid dendritic cells	39	29	700	56	63	521	442	226
Epidermal/Langerhans dendritic cells	43	271	1256	155	1910	469	1293	48
Red pulp macrophages	36	40	324	58	110	703	469	45
Peritoneal macrophages thioglycolate-elicited	43	102	744	88	1426	1980	575	44
Classical monocytes, MHCII−	47	26	652	64	47	3282	1346	110
Classical monocytes, MHCII+	51	40	767	68	63	2657	1275	72
Neutrophils	54	37	373	334	63	4298	841	59
Splenic natural killer cells	52	30	278	74	346	2110	575	63
Splenic natural killer cells Ly49CI− subset	51	36	300	76	457	1797	522	58
Splenic natural killer cells Ly49CI+ subset	50	37	232	72	346	1980	566	63
Doble-positive, small resting	49	30	334	78	63	234	381	51
CD4 single-positive, mature	44	23	140	70	94	104	899	61
CD8 single-positive, mature	43	26	140	56	63	339	1027	58
Spleen naive CD4	39	22	117	60	94	68	761	59
Spleen memory-phenotype CD4	42	31	258	72	520	781	788	95
Peyers patches naive CD4	52	33	122	70	63	52	504	46
Spleen CD25+ Tregs	53	34	301	78	510	287	611	79
Spleen naive CD8	47	26	140	66	63	391	619	59
Subcutaneous lymph nodes memory-phenotype CD8	57	37	163	102	79	1131	566	63
Thymic TCR $\gamma\delta$, all DN	44	27	168	62	220	208	673	58
Spleen TCR $\gamma\delta$, all DN	57	42	177	72	268	5343	535	60

Heat map generated with data from: http://www.immgen.org/.

Myo1c in B cells

When our group became interested in the presence and function of class I myosins in B lymphocytes, both Myo1e and Myo1g had been reported to be present in immune cells and, in particular, in B-cell lines (38, 39). Thus, although Myo1c has been extensively studied in different cell systems and is associated with such exquisite roles as mechanotransduction signaling at the inner ear (40) or transcription regulation and chromosome territories establishment (41), there was not a single description for the role of this myosin in leukocytes.

The first data that led us to suspect a possible function for Myo1c in B-cell biology were the early observations showing the presence of this protein in activated B lymphocytes and its polarization toward the dendrite-like extensions formed during the cell spreading process (5). This phenomenon could be induced by immobilized anti-CD44, B220, LFA-1, or MHC-II antibodies (2, 42) and has been associated with cell migration, activation, and antigen uptake (23). Further characterization of the expression patterns of Myo1c indicated that this molecular motor is indeed present at the different types of plasma membrane protrusions, such as filopodia and lamellipodia, that B lymphocytes develop during the spreading response (11). In addition, it was observed that Myo1c is not only present but also enriched at the microvilli (11).

Microvilli are protrusions present over the surface of lymphocytes, formed by a central F-actin bundle connected and supported by linking proteins to the plasma membrane (10, 43), that differ in structure and function from lamellipodia and filopodia. Microvilli are known to be enhanced in length and number upon activation of resting B cells (10, 11).

Epithelial cell microvilli are analogous to the microvilli in lymphocytes. In intestine and airway epithelial cells, class I myosins such as Myo1a promote the localization and stabilization of transmembrane molecules to certain areas of the plasma membrane, including microvilli (12, 44). Since

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enterocyte Myo1a binds and controls the movement of enzyme-containing lipid rafts to the tips of the microvilli (44), it seemed possible that Myo1c could perform a similar function in B cells. By using various approaches, including colocalization analyses, Förster resonance energy transfer (FRET) techniques, and immunoprecipitation, we could ascertain that Myo1c is indeed associated with the lipid rafts present in the B-cell plasma membrane, including the portion that constitutes the microvilli (11); these data support the hypothesis that Myo1c participates in the segregation of receptors, adhesion molecules, or signaling proteins in B lymphocytes.

Data from our group and others support the idea of microvilli as protein/membrane-domain segregating devices. Accordingly, the accumulation of different transmembrane proteins have been described in these structures, including the adhesion proteins CD44, L-selectin, LFA-1, and ICAM-1, costimulatory molecules CD40 and B7.2 (CD86), and ion channels CD20, CLIC1, and CLIC4 (10, 45–47).

Myo1c in B-lymphocyte antigen presentation

One of the most outstanding observations reported was that MHC-II are also enriched in B-cell microvilli (11, 47). These proteins, in conjunction with antigenic peptides, allow the activation of T cells upon contact with antigen-presenting cells (APCs); thus, MHC-II molecules represent key elements for the antigen presentation process in B lymphocytes and other APCs. MHC-II molecules have been thought to be immersed in lipid rafts (or detergent-resistant membranes), and their position inside these microdomains allows them to initiate signaling events implicated in maturation, proliferation, or cytokine production in B cells upon cross-linking induced by monoclonal antibodies or cognate T cells, resulting in transient Ca^{+2} release and recruitment/activation of Syk and Src family tyrosine kinases (48, 49).

Since both MHC-II molecules and Myo1c are present and enriched in B-cell microvilli, it is not surprising that these two proteins could be interacting at the plasma membrane; by using FRET and immunoprecipitation, it was shown that both molecules are in close contact, at least by an indirect interaction mediated by the scaffold that represent the lipid rafts (11).

Cross-linking experiments, performed using a monoclonal antibody, provide further evidence for interaction between MHC-II molecules and Myo1c. When the antibody is added to pre-activated B lymphocytes, which exhibit abundant and long microvilli, the above-mentioned signaling pathways are triggered, generating the collapse of the microvilli as shown in *Fig. 4A*. Interestingly, the coalescence of the MHC-II molecules induced by the cross-linking antibody correlates with a clear accumulation of Myo1c in the same membrane spot, a phenomenon observed as a high level of colocalization by confocal microscopy and named 'co-capping' (11). Since the cross-linking by the anti-MHC-II antibody in these experiments resembles that induced by contact with a T lymphocyte, we further investigated whether something similar could also occur during the immunological synapse (IS).

The contact between any APC and a T lymphocyte is commonly referred as IS. Briefly, during this process, the MHC-peptide complexes displayed over the surface of the APC are recognized by a cognate T-cell receptor (TCR) to activate a corresponding T-cell clone. This process involves complex temporal and spatial regulation of protein complexes to coordinate and tune signaling events (50–52). As expected, and in concordance with the co-capping data, Myo1c was found to accumulate at the B-cell side during IS formation with T cells (11). This observation is highly important, since some groups have previously investigated the participation of leukocyte myosins such as MyH9 in the organization and clustering of membrane receptors, such as the TCR, during IS formation. Some authors have reported that MyH9 regulates retrograde flow but is not required for proper synapse assembly (17). Other reports suggest that this myosin is necessary for centripetal motion and fusion of signaling microclusters (53); however, they also indicate that the accumulation of actin, ezrin, and the TCR at the IS was not altered after the inhibition of MyH9. Consequently, Myo1c emerges as a candidate protein for active transport of membrane components to the IS interface.

Since we propose an organizational and tethering role for Myo1c at the APC side of the IS, the obvious consequence of inhibiting its functions should be an alteration in antigen presentation. The introduction of a dominant negative form of Myo1c (lacking the motor domain) or the downregulation of Myo1c expression with specific siRNA was found to generate a diminished antigen presentation ability in the affected B cells, as determined by measuring IL-2 secreted by activated T cells in co-culture (11).

The same B cells transfected with a dominant negative form of Myo1c lack the ability to cluster MHC-II molecules after cross-linking induced by the specific antibody (*Fig. 4B and C*). In addition, these same B cells exhibit a reduced spreading response compared with control cells (11).

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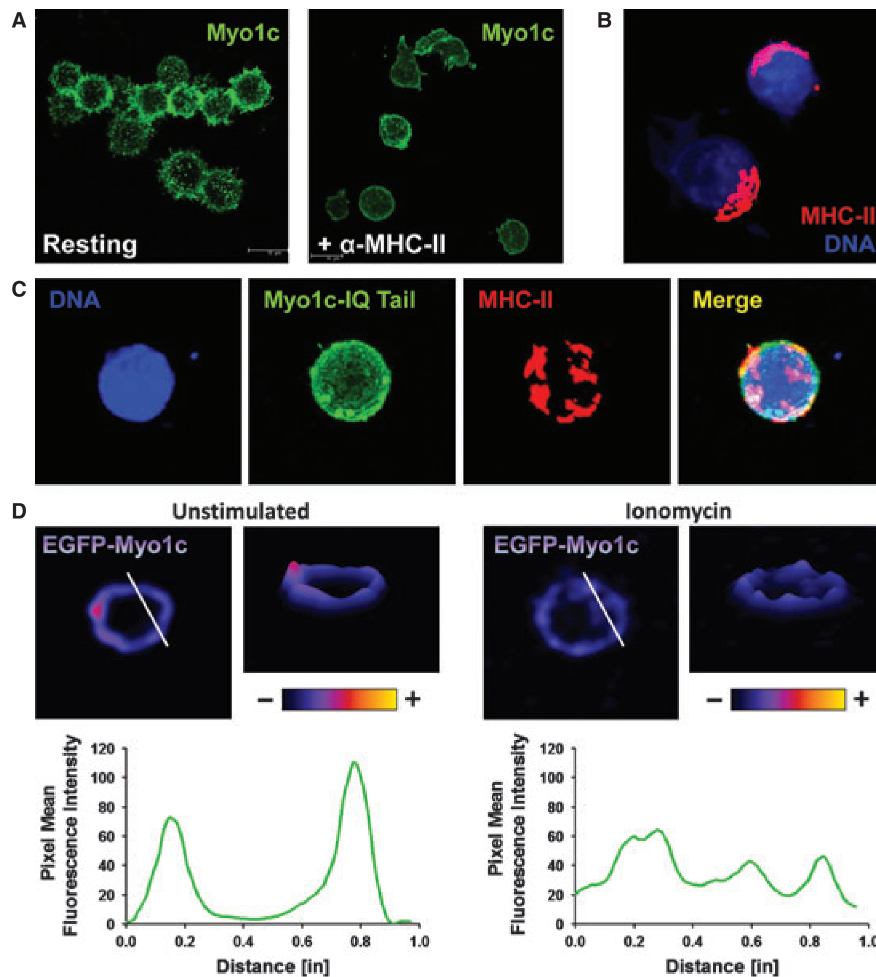

Fig. 4. Myosin 1c in B lymphocytes. (A). Presence of Myo1c (stained in green) in the B-cell microvilli at the plasma membrane; interestingly, when previously lipopolysaccharide+IL-4 activated cells showing long and abundant microvilli (left panel) are stimulated with anti-MHC-II, the microvilli collapse (right panel). (B). Induction of MHC-II clustering by a cross-linking anti-MHC-II; the 'cap' of histocompatibility molecules (in red) is clearly observed at one pole of the cells. (C). The expression of a dominant negative-form of Myo1c (Myo1c IQ-Tail, tagged with EGFP) abolished the MHC-II capping ability of B cells. (D). EGFP-tagged Myo1c was transduced into primary B cells to detect the membrane-associated distribution of Myo1c, showed as a spectral 3D view indicating the concentration of the protein according to the color scales displayed (upper left panels). Time-lapse experiments using live cells showed that the addition of a Ca^{+2} ionophore (ionomycin) alters Myo1c distribution that becomes partially cytoplasmic (upper right panels). This is better observed when the mean fluorescence intensity of the pixels depicted by the white line over the spectral images was calculated and graphed versus the length of mentioned line. In these graphs (lower panels) we can see that the resting cell display two highly fluorescent peaks that correspond to the intersection points of the line with the plasma membrane. After the addition of ionomycin the height of these peaks drops and it is possible to observe fluorescent pixels between these membrane borders, which correspond to cytoplasmic tagged-Myo1c.

The evidence discussed above indicates that Myo1c possesses a dual role in B lymphocytes. First, this molecular motor functions as an anchor in structures such as the microvilli; here, the myosin could be promoting the segregation of the membrane domains, represented by lipid rafts, including proteins such as MHC-II (among others). It is also possible that Myo1c, with its ability to link both the plasma membrane and the cytoskeleton, could promote the spatial homogeneous distribution of these domains during the 'steady state' of the cell in the same way as other linkage proteins in B lymphocytes, such as ezrin (54, 55). This activity could be responsible for inhibiting the spontaneous coalescence of the lipid rafts and the signaling proteins that they contain under resting conditions, as is supported by different observations claiming a direct role for cytoskeleton filaments and their associated proteins in the determination of membrane topology (54–61).

Second, after the generation of activation signals such as Ca^{+2} fluxes and kinase recruitment induced by IS formation, the accumulation of Myo1c in the B cell toward the focal contact zone coincides with a fast F-actin polymerization under the contact zone (62). As the polarization of PI(4,5)

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P_2 (63) (the phosphoinositide which binds to the Myo1c TH domain) and MHC-II have been also detected in this region, it is possible that Myo1c helps to stabilize the agglomeration of membrane signaling complexes required at the APC-T-cell interface; however, there is also the possibility that Myo1c, with its motor activity, could be actively helping in the recruitment of these signaling complexes via the agglutination of lipid rafts in that area. This latter hypothesis may be supported by preliminary observations that after a Ca^{+2} flux, such as the ones resulting from the stimulation of a B cell through its BCR or via integrin activation, the membrane distribution of Myo1c is altered, since it can be observed as soluble protein in the cytoplasm (*Fig. 4D*). This alteration in distribution could be generated both by changes in the Myo1c light chain affinity (which is regulated by Ca^{+2} cytoplasmic concentrations) or via the transient F-actin depolymerization induced by activating signals.

Myo1g in B cells

Myo1g has been identified as a myosin exclusive to hematopoietic cells (29, 37, 64), where its expression levels vary between the different lineages, being especially abundant in B cells and activated T cells. Mass spectrometric proteomic profiling of lymphocyte proteins indicates that Myo1g is actually the most abundant class I myosin expressed by T lymphocytes (39), in which, as observed by immunofluorescence assays, it localizes to the plasma membrane, is particularly enriched at cell surface microvilli, and is associated in an ATP-releasable manner to the actin cytoskeleton (39, 64).

Although there are currently very few reports concerning the role of Myo1g, it is known that this motor protein is involved in the maintenance and regulation of plasma membrane tension in cells where is expressed. It has been shown, by using specific siRNA for Myo1g, that the elasticity of the T-cell membrane is diminished in response to this motor protein deficiency (64).

As described earlier, Myo1g is also present in B lymphocytes (11, 39) (*Table 1*). In the experiments discussed above in which Myo1c was knocked down, it was possible to observe defects in lymphocyte migration and cytoskeleton rearrangements during cell spreading (11). This observation suggests that Myo1g could also participate in these processes by regulating membrane dynamics.

Myo1g was reported to be found associated with the plasma membrane of lymphocytes through its TH1 region (39). We detected the presence of Myo1g at the microvilli protrusions of spleen B cells activated for 48 h with lipopolysaccharide and IL-4 and confirmed the abundance of Myo1g in these microstructures (*Fig. 5A*). Examination of B lymphocytes spread over an anti-CD44-coated surface showed that Myo1g is located in adhesion/motility-related protrusions, such as actin-dependent lamellipodia and filopodia, and is also visible at the longer highly dynamic dendrite-like protrusions (*Fig. 5B*).

Possible roles of Myo1g in vesicular traffic

Myo1g has been shown to bind to $PI(4,5)P_2$ (39) and phosphatidylinositol 3,4,5trisphosphate (65) and thus may have a role in the vesicular transportation of endosomes. Several endosomes have a rich concentration of these lipids, such as endosomes in HeLa cells (66) and recycling endosomes in epithelial cells (67). On the other hand, Myo1g has been found in lipid rafts from neutrophils (68), in human T lymphocytes (69), and in fractions containing lipid rafts from late endosomes derived from baby hamster kidney cells (70). Lipid rafts are microdomains that are present in the plasma membrane; these microdomains facilitate the formation of clusters for signaling. Thus, these microdomains play a critical role in signaling via membrane receptors. These receptors initiate the recruitment and assembly of more signaling subunits, forming supramolecular complexes that regulate signal transduction (71). Therefore, we investigated whether Myo1g was present in these microdomains. Myo1g was found in lipid rafts purified by sucrose gradients from activated B lymphocytes; rafts were identified by the presence of flotillin and the ganglioside GM1 (J. L. Maravillas-Montero, manuscript in preparation). Thus, Myo1g may help in recruiting receptors and signaling molecules to establish supramolecular signaling complexes that induce the activation and differentiation of B cells.

Lipid rafts have been implicated in the vesicular traffic of several types of proteins, such as integrins (72, 73), CD44 (74), MHC-II (75), CD55, and CD59 (76), among others. The model of transportation based on these microdomains implies that the mechanism of apical transport is based on lipid-lipid and lipid-protein interactions. These events occur in specific microdomains with a high content of cholesterol and glycolipids. Glycosphingolipids are associated with and form clusters on the membranes of the trans-Golgi network (TGN). These clusters may act as platforms for the inclusion of protein 'loads' intended to be transported to the apical membrane, whereas proteins destined for the basolateral

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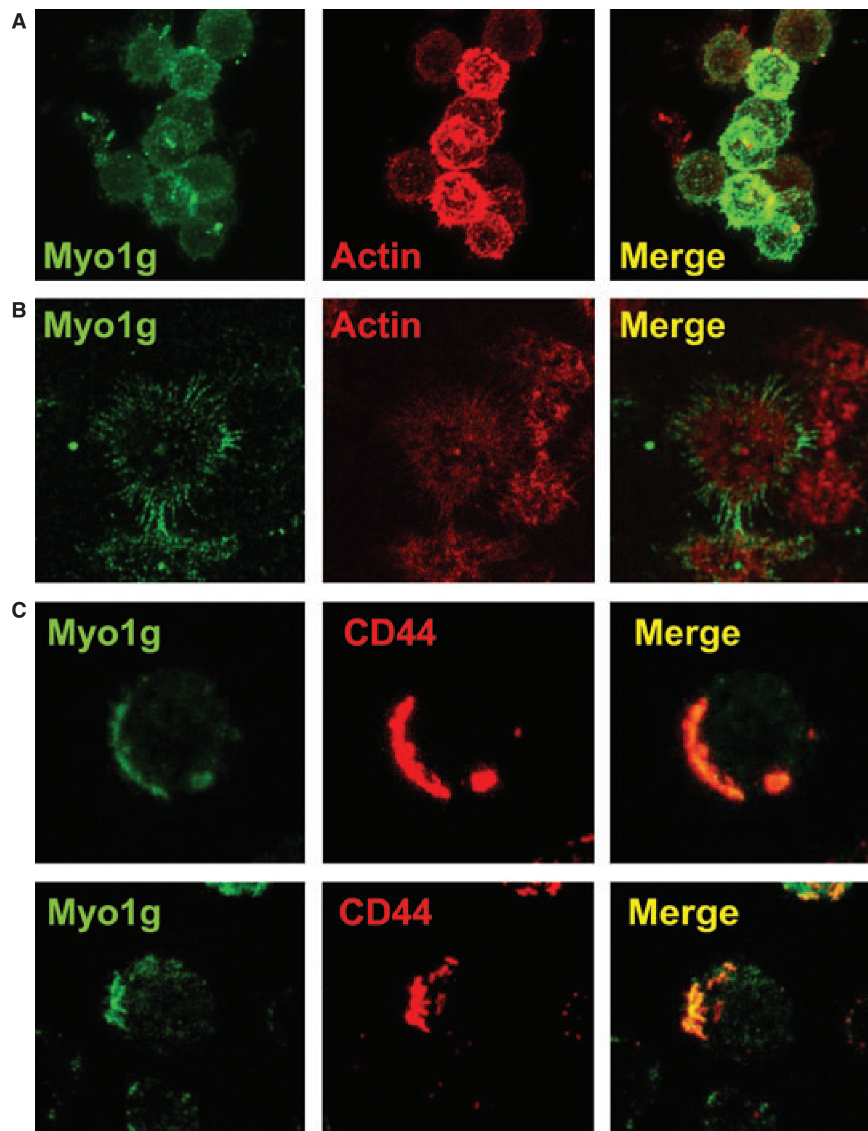

Fig. 5. Myosin 1g in B lymphocytes is also present at membrane extensions and is polarized during capping. (A). Activated B cells displaying multiple microvilli over their surface were stained with rhodamine-phalloidin (red) and anti-Myo1g (green). (B). Spread B lymphocytes over a CD44-coated plate were stained for F-actin (red) and the anti-Myo1g (green) to detect the thin dendrite like extensions typically observed during this phenomenon. (C). Myo1g (green) polarization after anti-CD44 (red) induced capping in primary B cells.

membrane are excluded. The lipids and protein loads could be assembled, together with accessory proteins, on the cytoplasmic face of the TGN to create a subdomain able to form vesicles containing the apparatus necessary to ensure a specific distribution to the apical membrane (77). In rounded (not polarized) cells such as lymphocytes, the traffic of molecules can also have a directed transport. For example, the interaction between B cells (acting as APCs) and T cells promotes the concentration of specific receptor sat the interface of the IS. Secretion by T cells has also been shown to have this directional-specific transport to the site of contact: secretion of IL-2 and IFN-γ by CD4^{+} T cells or perforin/granzyme granules by CD8^{+} T cells is directed to the site of contact with the target cells to maximize its function (78).

Myo1g in the IS

One of the main functions of activated B lymphocytes is the presentation of antigen to T lymphocytes to receive costimulation (via cytokines and other molecules). Antigen presentation is realized by the IS, a specialized structure that promotes mobilization of MHC molecules (79, 80) and adhesion proteins (81). Within this interface [also called the supramolecular activation cluster (SMAC)], three regions have been determined (82); these regions are defined

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according to the distribution of the proteins recruited to and sorted within the IS. The most central cluster (cSMAC) is characterized by the presence of the TCR, MHC (82, 83), CD28 (84), and costimulatory molecules (85), among others. The peripheral cluster (pSMAC) is defined by the distribution of adhesion molecules such as LFA-1 (83) and ICAM1 (86), and a more distal cluster (dSMAC) is determined by the localization of CD45 (83). CD44 has also been found polarized to IS in the APC (87). Myosins contribute in the mobilization of several molecules to the IS; for example, MyH9 participates in the maturation of this structure. The inhibition of MyH9 by siRNA in T lymphocytes has been shown to increase spreading; however, these cells are unable to assemble cSMAC and pSMAC. In addition, these cells exhibited a decrease in phosphorylation of Src kinase and Cas-L (19, 88). The increase in spreading may be a consequence of the interaction between MyH9 and LFA-1 in T lymphocytes. Interaction between MyH9 and LFA-1 has been observed at the uropod of CXCL12-stimulated T lymphocytes. Pharmacological inhibition of MyH9 results in extreme uropod elongation and decreased lymphocyte migration because the treated T lymphocytes have defective tail detachment (19).

We have shown that Myo1c participates in B-cell cytoskeleton rearrangements and is recruited to the IS, contributing to antigen presentation. Because some Myo1g is also recruited into lipid rafts, it is possible that it may also participate in the generation of the IS. In addition, we have shown that cross-linking of CD44, ICAM-1, and LFA-1 induces the reorganization of Myo1g, indicating that it may participate in the mobilization or recruitment of these molecules during IS formation (Fig. 5C).

Concluding remarks

The study of B-cell spreading has allowed us to analyze aspects of the cytoskeleton. Although we as yet do not have a clear idea of the functional meaning of spreading, one of the functions that has been hypothesized to explain this phenomenon is that the extension of the membrane allows the sensing of a larger area in search of antigens (23), which involves several types of molecules, ranging from signaling molecules, cytoskeleton-associated proteins, and receptors, among others.

The dramatic changes in the morphology of B lymphocytes have fascinated us from the very beginning. We believe that the plasticity of the B-cell membrane as seen during spreading may be equivalent to phenomena such as migration and cell-cell interaction, because there is a dramatic reorganization of several surface molecules, and different components of the cytoskeleton actively participate to produce all the changes we have described above. In any case, the analyses of more canonical interactions, such as the formation of IS, has clearly shown that APCs, such as B cells, actively participate in the phenomena and are not merely passive objects of T-cell activation. New areas of analysis have been opened in our laboratory as a result of the analysis of the cytoskeleton and, in recent years, more specifically, of the role of class I myosins. We have shown the role of Myo1c in antigen presentation, IS formation, and migration. Similar results are being obtained from the analysis of Myo1g, where we have preliminary data showing that this molecule may be also involved in spreading, cell migration, phagocytosis, endocytosis, and exocytosis (O. López-Ortega, manuscript in preparation). In summary, class I myosins are important in several functions of B lymphocytes, and recent data confirm some of our initial suspicions about the role of these proteins in the biology of B lymphocytes.

References

1. Cambier JC, Lehmann KR. Ia-mediated signal transduction leads to proliferation of primed B lymphocytes. J Exp Med 1989;**170**:877–886.
2. Partida-Sanchez S, Garibay-Escobar A, Frixione E, Parkhouse RM, Santos-Argumedo L. CD45R, CD44 and MHC class II are signaling molecules for the cytoskeleton-dependent induction of dendrites and motility in activated B cells. Eur J Immunol 2000;**30**:2722–2778.
3. Santos-Argumedo L, Kincade PW, Partida-Sanchez S, Parkhouse RM. CD44-stimulated dendrite formation ('spreading') in activated B cells. Immunology 1997;**90**:147–153.
4. Sumoza-Toledo A, Santos-Argumedo L. The spreading of B lymphocytes induced by CD44 cross-linking requires actin, tubulin, and vimentin rearrangements. J Leukoc Biol 2004;**75**:233–239.
5. Sumoza-Toledo A, Gillespie PG, Romero-Ramirez H, Ferreira-Ishikawa HC, Larson RE, Santos-Argumedo L. Differential localization of unconventional myosin I and nonmuscle myosin II during B cell spreading. Exp Cell Res 2006;**312**: 3312–3322.
6. Davey EJ, Greicius G, Thyberg J, Severinson E. STAT6 is required for the regulation of IL-4-induced cytoskeletal events in B cells. Int Immunol 2000;**12**:995–1003.
7. Zohar R, et al. Intracellular osteopontin is an integral component of the CD44-ERM complex involved in cell migration. J Cell Physiol 2000;**184**:118–130.
8. Tsukita S, Oishi K, Sato N, Sagara J, Kawai A. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J Cell Biol 1994;**126**:391–401.
9. Legg JW, Isacke CM. Identification and functional analysis of the ezrin-binding site in the hyaluronan receptor, CD44. Curr Biol 1998;**8**:705–708.

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10. Greicius G, et al. Microvilli structures on B lymphocytes: inducible functional domains? Int Immunol 2004;**16**:353–364.
11. Maravillas-Montero JL, Gillespie PG, Patino-Lopez G, Shaw S, Santos-Argumedo L. Myosin 1c participates in B cell cytoskeleton rearrangements, is recruited to the immunologic synapse, and contributes to antigen presentation. J Immunol 2011;**187**:3053–3063.
12. Tyska MJ, Mackey AT, Huang JD, Copeland NG, Jenkins NA, Mooseker MS. Myosin-1a is critical for normal brush border structure and composition. Mol Biol Cell 2005;**16**:2443–2457.
13. De La Cruz EM, Ostap EM. Relating biochemistry and function in the myosin superfamily. Curr Opin Cell Biol 2004;**16**:61–67.
14. Mermall V, Post PL, Mooseker MS. Unconventional myosins in cell movement, membrane traffic, and signal transduction. Science 1998;**279**: 527–533.
15. Krendel M, Mooseker MS. Myosins: tails (and heads) of functional diversity. Physiology 2005;**20**:239–251.
16. Richards TA, Cavalier-Smith T. Myosin domain evolution and the primary divergence of eukaryotes. Nature 2005;**436**:1113–1118.
17. Jacobelli J, Chmura SA, Buxton DB, Davis MM, Krummel MF. A single class II myosin modulates T cell motility and stopping, but not synapse formation. Nat Immunol 2004;**5**:531–538.
18. Kim SV, et al. Modulation of cell adhesion and motility in the immune system by Myo1f. Science 2006;**314**:136–139.
19. Morin NA, et al. Nonmuscle myosin heavy chain IIA mediates integrin LFA-1 de-adhesion during T lymphocyte migration. J Exp Med 2008;**205**: 195–205.
20. Rey M, et al. Cutting edge: association of the motor protein nonmuscle myosin heavy chain-IIA with the C terminus of the chemokine receptor CXCR4 in T lymphocytes. J Immunol 2002;**169**:5410–5414.
21. Vascotto F, et al. The actin-based motor protein myosin II regulates MHC class II trafficking and BCR-driven antigen presentation. J Cell Biol 2007;**176**:1007–1019.
22. Natkanski E, Lee WY, Mistry B, Casal A, Molloy JE, Tolar P. B cells use mechanical energy to discriminate antigen affinities. Science 2013;**340**:1587–1590.
23. Fleire SJ, Goldman JP, Carrasco YR, Weber M, Bray D, Batista FD. B cell ligand discrimination through a spreading and contraction response. Science 2006;**312**:738–741.
24. Coluccio LM, Myosin I, Am J. Physiol 1997;**273**: C347–C359.
25. Kim SV, Flavell RA. Myosin I: from yeast to human. Cell Mol Life Sci 2008;**65**:2128–2137.
26. Albanesi JP, Fujisaki H, Hammer JA 3rd, Korn ED, Jones R, Sheetz MP. Monomeric Acanthamoeba myosins I support movement in vitro. J Biol Chem 1985;**260**:8649–8652.
27. Stafford WF, Walker ML, Trinick JA, Coluccio LM. Mammalian class I myosin, Myo1b, is monomeric and cross-links actin filaments as determined by hydrodynamic studies and electron microscopy. Biophys J 2005;**88**:384–391.
28. Cheney RE, Mooseker MS. Unconventional myosins. Curr Opin Cell Biol 1992;**4**: 27–35.
29. Maravillas-Montero JL, Santos-Argumedo L. The myosin family: unconventional roles of actin-dependent molecular motors in immune cells. J Leukoc Biol 2012;**91**:35–46.
30. Hammer JA. Novel myosins. Trends Cell Biol 1991;**1**:50–56.
31. Doberstein SK, Pollard TD. Localization and specificity of the phospholipid and actin binding sites on the tail of Acanthamoeba myosin IC. J Cell Biol 1992;**117**:1241–1249.
32. Lee WL, Ostap EM, Zot HG, Pollard TD. Organization and ligand binding properties of the tail of Acanthamoeba myosin-IA. Identification of an actin-binding site in the basic (tail homology-1) domain. J Biol Chem 1999;**274**:35159–35171.
33. Jung G, Hammer JA 3rd. The actin binding site in the tail domain of Dictyostelium myosin IC (myoC) resides within the glycine- and proline-rich sequence (tail homology region 2). FEBS Lett 1994;**342**:197–202.
34. Rump A, et al. Myosin-1C associates with microtubules and stabilizes the mitotic spindle during cell division. J Cell Sci 2011;**124**:2521–2528.
35. Lynch TJ, Albanesi JP, Korn ED, Robinson EA, Bowers B, Fujisaki H. ATPase activities and actin-binding properties of subfragments of Acanthamoeba myosin IA. J Biol Chem 1986;**261**:17156–17162.
36. Berg JS, Powell BC, Cheney RE. A millennial myosin census. Mol Biol Cell 2001;**12**:780–794.
37. Heng TS, Painter MW. The Immunological Genome Project: networks of gene expression in immune cells. Nat Immunol 2008;**9**:1091–1094.
38. Lindvall JM, Blomberg KE, Wennborg A, Smith CI. Differential expression and molecular characterisation of Lmo7, Myo1e, Sash1, and Mcoln2 genes in Btk-defective B-cells. Cell Immunol 2005;**235**:46–55.
39. Patino-Lopez G, Aravind L, Dong X, Kruhlak MJ, Ostap EM, Shaw S. Myosin 1G is an abundant class I myosin in lymphocytes whose localization at the plasma membrane depends on its ancient divergent pleckstrin homology (PH) domain (Myo1PH). J Biol Chem 2010;**285**:8675–8686.
40. Gillespie PG, Cyr JL. Myosin-1c, the hair cell's adaptation motor. Annu Rev Physiol 2004;**66**:521–545.
41. Hofmann WA, Johnson T, Klapczynski M, Fan JL, de Lanerolle P. From transcription to transport: emerging roles for nuclear myosin I. Biochem Cell Biol 2006;**84**:418–426.
42. Lin KB, et al. The rap GTPases regulate B cell morphology, immune-synapse formation, and signaling by particulate B cell receptor ligands. Immunity 2008;**28**:75–87.
43. McConnell RE, Higginbotham JN, Shifrin DA Jr, Tabb DL, Coffey RJ, Tyska MJ. The enterocyte microvillus is a vesicle-generating organelle. J Cell Biol 2009;**185**:1285–1298.
44. Tyska MJ, Mooseker MS. A role for myosin-1A in the localization of a brush border disaccharidase. J Cell Biol 2004;**165**:395–405.
45. Abitorabi MA, Pachynski RK, Ferrando RE, Tidswell M, Erle DJ. Presentation of integrins on leukocyte microvilli: a role for the extracellular domain in determining membrane localization. J Cell Biol 1997;**139**:563–571.
46. Steegmaier M, Borges E, Berger J, Schwarz H, Vestweber D. The E-selectin-ligand ESL-1 is located in the Golgi as well as on microvilli on the cell surface. J Cell Sci 1997;**110**:687–694.
47. Hao JJ, et al. Enrichment of distinct microfilament-associated and GTP-binding-proteins in membrane/microvilli fractions from lymphoid cells. J Proteome Res 2008;**7**:2911–2927.
48. Al-Daccak R, Mooney N, Charron D. MHC class II signaling in antigen-presenting cells. Curr Opin Immunol 2004;**16**:108–113.
49. Gombos I, Detre C, Vamosi G, Matko J. Rafting MHC-II domains in the APC (presynaptic) plasma membrane and the thresholds for T-cell activation and immunological synapse formation. Immunol Lett 2004;**92**:117–124.
50. Batista FD, Saito T. The immunological synapse enters its second decade. Curr Top Microbiol Immunol 2010;**340**:ix–x.
51. Friedl P, Storim J. Diversity in immune-cell interactions: states and functions of the immunological synapse. Trends Cell Biol 2004;**14**:557–567.
52. Lee KH, et al. The immunological synapse balances T cell receptor signaling and degradation. Science 2003;**302**:1218–1222.
53. Ilani T, Vasiliver-Shamis G, Vardhana S, Bretscher A, Dustin ML. T cell antigen receptor signaling and immunological synapse stability require myosin IIA. Nat Immunol 2009;**10**: 531–539.
54. Gupta N, Wollscheid B, Watts JD, Scheer B, Aebersold R, DeFranco AL. Quantitative proteomic analysis of B cell lipid rafts reveals that ezrin regulates antigen receptor-mediated lipid raft dynamics. Nat Immunol 2006;**7**:625–633.
55. Treanor B, et al. The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor. Immunity 2010;**32**:187–199.
56. Batista FD, Treanor B, Harwood NE. Visualizing a role for the actin cytoskeleton in the regulation of B-cell activation. Immunol Rev 2010;**237**:191–204.
57. Ben-Aissa K, et al. Activation of moesin, a protein that links actin cytoskeleton to the plasma membrane, occurs by phosphatidylinositol 4,5-bisphosphate (PIP2) binding sequentially to two sites and releasing an autoinhibitory linker. J Biol Chem 2012;**287**:16311–16323.
58. Burkhardt JK, Carrizosa E, Shaffer MH. The actin cytoskeleton in T cell activation. Annu Rev Immunol 2008;**26**:233–259.
59. Das V, et al. Membrane-cytoskeleton interactions during the formation of the immunological synapse and subsequent T-cell activation. Immunol Rev 2002;**189**:123–135.
60. Geppert TD, Lipsky PE. Association of various T cell-surface molecules with the cytoskeleton. Effect of cross-linking and activation. J Immunol 1991;**146**:3298–3305.
61. Gordy C, Mishra S, Rodgers W. Visualization of antigen presentation by actin-mediated targeting of glycolipid-enriched membrane domains to the

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immune synapse of B cell APCs. J Immunol 2004;**172**:2030–2038.
62. Harwood NE, Batista FD. Early events in B cell activation. Annu Rev Immunol 2010;**28**:185–210.
63. Fooksman DR, Shaikh SR, Boyle S, Edidin M. Cutting edge: phosphatidylinositol 4,5-bisphosphate concentration at the APC side of the immunological synapse is required for effector T cell function. J Immunol 2009;**182**:5179–5182.
64. Olety B, Walte M, Honnert U, Schillers H, Bahler M. Myosin 1G (Myo1G) is a haematopoietic specific myosin that localises to the plasma membrane and regulates cell elasticity. FEBS Lett 2010;**584**:493–499.
65. Dart AE, Tollis S, Bright MD, Frankel G, Endres RG. The motor protein myosin 1G functions in FcgammaR-mediated phagocytosis. J Cell Sci 2012;**125**:6020–6029.
66. Shi A, et al. RAB-10-GTPase-mediated regulation of endosomal phosphatidylinositol-4,5-bisphosphate. Proc Natl Acad Sci USA 2012;**109**:E2306–E2315.
67. Fields IC, King SM, Shteyn E, Kang RS, Folsch H. Phosphatidylinositol 3,4,5-trisphosphate localization in recycling endosomes is necessary for AP-1B-dependent sorting in polarized epithelial cells. Mol Biol Cell 2010;**21**:95–105.
68. Nebl T, Pestonjamasp KN, Leszyk JD, Crowley JL, Oh SW, Luna EJ. Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes. J Biol Chem 2002;**277**:43399–43409.
69. Linkermann A, Gelhaus C, Lettau M, Qian J, Kabelitz D, Janssen O. Identification of interaction partners for individual SH3 domains of Fas ligand associated members of the PCH protein family in T lymphocytes. Biochim Biophys Acta 2009;**1794**:168–176.
70. Lafourcade C, Sobo K, Kieffer-Jaquinod S, Garin J, van der Goot FG. Regulation of the V-ATPase along the endocytic pathway occurs through reversible subunit association and membrane localization. PLoS ONE 2008;**3**:e2758.
71. Guo B, Kato RM, Garcia-Lloret M, Wahl MI, Rawlings DJ. Engagement of the human pre-B cell receptor generates a lipid raft-dependent calcium signaling complex. Immunity 2000;**13**:243–253.
72. Fabbri M, et al. Dynamic partitioning into lipid rafts controls the endo-exocytic cycle of the alphaL/beta2 integrin, LFA-1, during leukocyte chemotaxis. Mol Biol Cell 2005;**16**:5793–5803.
73. Wang C, Yoo Y, Fan H, Kim E, Guan KL, Guan JL. Regulation of Integrin beta 1 recycling to lipid rafts by Rab1a to promote cell migration. J Biol Chem 2010;**285**:29398–29405.
74. Thankamony SP, Knudson W. Acylation of CD44 and its association with lipid rafts are required for receptor and hyaluronan endocytosis. J Biol Chem 2006;**281**:34601–34609.
75. Knorr R, Karacsonyi C, Lindner R. Endocytosis of MHC molecules by distinct membrane rafts. J Cell Sci 2009;**122**:1584–1594.
76. Brandstaetter H, Kendrick-Jones J, Buss F. Myo1c regulates lipid raft recycling to control cell spreading, migration and Salmonella invasion. J Cell Sci 2012;**125**: 1991–2003.
77. Wandinger-Ness A, Bennett MK, Antony C, Simons K. Distinct transport vesicles mediate the delivery of plasma membrane proteins to the apical and basolateral domains of MDCK cells. J Cell Biol 1990;**111**:987–1000.
78. Purbhoo MA. The function of sub-synaptic vesicles during T-cell activation. Immunol Rev 2013;**251**:36–48.
79. Norcross MA. A synaptic basis for T-lymphocyte activation. Ann Immunol 1984;**135D**:113–134.
80. Paul WE, Seder RA. Lymphocyte responses and cytokines. Cell 1994;**76**:241–251.
81. Grakoui A, et al. The immunological synapse: a molecular machine controlling T cell activation. Science 1999;**285**:221–227.
82. Anton van der Merwe P, Davis SJ, Shaw AS, Dustin ML. Cytoskeletal polarization and redistribution of cell-surface molecules during T cell antigen recognition. Semin Immunol 2000;**12**:5–21.
83. Graf B, Bushnell T, Miller J. LFA-1-mediated T cell costimulation through increased localization of TCR/class II complexes to the central supramolecular activation cluster and exclusion of CD45 from the immunological synapse. J Immunol 2007;**179**:1616–1624.
84. Sanchez-Lockhart M, Graf B, Miller J. Signals and sequences that control CD28 localization to the central region of the immunological synapse. J Immunol 2008;**181**:7639–7648.
85. Tseng SY, Waite JC, Liu M, Vardhana S, Dustin ML. T cell-dendritic cell immunological synapses contain TCR-dependent CD28-CD80 clusters that recruit protein kinase C theta. J Immunol 2008;**181**:4852–4863.
86. Liu D, Bryceson YT, Meckel T, Vasiliver-Shamis G, Dustin ML, Long EO. Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic immune synapses. Immunity 2009;**31**:99–109.
87. Hegde VL, Singh NP, Nagarkatti PS, Nagarkatti M. CD44 mobilization in allogeneic dendritic cell-T cell immunological synapse plays a key role in T cell activation. J Leukoc Biol 2008;**84**:134–142.
88. Kumari S, et al. T lymphocyte Myosin IIA is required for maturation of the immunological synapse. Front Immunol 2012;**3**:230.

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