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REVIEW

Class I myosins: Highly versatile proteins with specific functions in the immune system

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Abstract

Connections established between cytoskeleton and plasma membrane are essential in cellular processes such as cell migration, vesicular trafficking, and cytokinesis. Class I myosins are motor proteins linking the actin-cytoskeleton with membrane phospholipids. Previous studies have implicated these molecules in cell functions including endocytosis, exocytosis, release of extracellular vesicles and the regulation of cell shape and membrane elasticity. In immune cells, those proteins also are involved in the formation and maintenance of immunological synapse-related signaling. Thus, these proteins are master regulators of actin cytoskeleton dynamics in different scenarios. Although the localization of class I myosins has been described in vertebrates, their functions, regulation, and mechanical properties are not very well understood. In this review, we focused on and summarized the current understanding of class I myosins in vertebrates with particular emphasis in leukocytes.

KEYWORDS

class I myosins, molecular motors, cytoskeleton, plasma membrane, leukocytes

1 | MYOSINS

Myosins are motor proteins characterized by their ability to bind to filamentous actin. These proteins hydrolyze ATP to induce conformational changes allowing their displacement along the actin.[1–4] Myosins are multimeric proteins formed by one or two heavy chains and a variable number of light chains. In heavy chains, the amino-terminal region is a globular head where two or more light chains are associated with and regulate myosin movement. The myosin head domain is the most preserved region. This region contains actin and ATP-binding sites which convert chemical energy into mechanical energy.[5] The motor protein is highly conserved. Cope and colleagues have shown, by the analysis of 13 different classes of myosins (82 different myosins), that the motor domains have at least 131 residues conserved in 90% of their sequences,[5] this result indicate a preserved function important for catalysis, recognition, and stability of the motor protein. The carboxyl-terminal portion (known as "tail") is the least conserved sequence and facilitates heavy chain dimerization, plasma membrane interactions and protein–protein associations.[6–9]

Many myosins exhibit the property of processivity (wherein the motor undergoes multiple catalytic cycles and coupled mechanical advances for each diffusional encounter with its track),[10] although most class I myosins are thought to be non-processive. When ATP binds to myosin, a conformational change is induced and myosin is released from F-actin, next when ATP is hydrolyzed (ADP + Pi), the myosin once again interacts with the actin. Finally, ADP is released, allowing ATP to bind to myosin to begin a new cycle.[10]

Myosin generates a driving force through the release of pyrophosphate, this release causes a conformational change in the head domain. The change in conformation produces the rotation of myosin that is important for displacement and for the association of myosin to F-actin. Different conformations of the motor myosin in the level arm are important for ATP release. Mutations or deletions in the N-terminal region (NTR) affect the association and release of pyrophosphate[11] which in turn affects the tension of myosins with filamentous actin.

The area between the head and the tail contains a domain known as "neck," which is formed by a variable number of IQ motifs and

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; BTK, Bruton's tyrosine kinase; Ca^{2+}, calcium ions; CamKII, calcium/calmodulin kinase II; EBV, Epstein–Barr virus; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; IQ, Isoleucine-glutamine; LPS, lipopolysaccharides; MHC-II, major histocompatibility complex class II; MLL, mixed lineage leukemia; Myo, myosin; PHA, phytohemagglutinin; Pi, inorganic phosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; SNP, single nucleotide polymorphism; VEFGR2, vascular endothelial growth factor receptor-2

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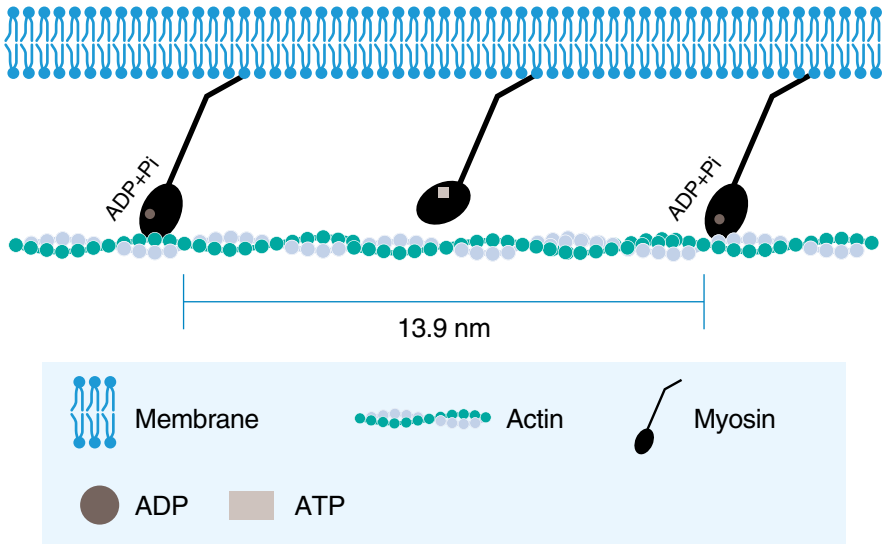

FIGURE 1 **Class I myosins, structure and motility.** Myosins utilize ATP hydrolysis to induce mechanical movement along actin filament. This figure shows a representation of attachment and detachment sequence of class I myosins on actin cytoskeleton. The process is regulated by ATP-ADP+Pi cycle. As an example, Myo1d is used to represent a 13.9 nm step size[21]

is characterized by the IQXXXRGXXXR sequence (I-isoleucine; Q-glutamine).[12,13] The IQ motif, functions as a binding site for calmodulin (CaM), and CaM-like proteins.[14]

2 | CLASS I MYOSINS

Class I myosins are monomeric proteins involved in actin polymerization, cell motility, cell shape control, sensory transduction, and vesicular trafficking.[15,16] They were initially described in *Acanthamoeba* in 1973.[17] Being monomers, they were referred to as "Class I" myosins as opposed to conventional myosins which are homodimers (or "Class II"). However, a large number of unconventional myosins (both dimeric and monomeric) has been discovered.[6,8,18,19] Class I myosins are monomeric proteins, the movement of a single myosin along polymerized actin or step size (displacement produced during one ATPase cycle) varies in accordance with lever arm length and lever arm rotation degree. The rotation of the lever arm, cause changes in the step size. For example, Greenberg and colleagues reported that mouse Myo1c have a step size of 7.8±13 nm,[20] while the work of Köhler and colleagues showed that rat Myo1d has a 13.9±0.15 nm step size (Fig. 1). However, when these measurements were made using only with the head domain of Myo1d, the step size decreased to 4.9± 0.21 nm.[21] These results indicate the tail domain might participate in the step size of class I myosins. Analysis by cryo-electron microscopy of Myo1b shows that this protein has two binding states to ADP, separated by a 25° lever arm; the first ADP state is important to open the nucleotide-binding site, the second state allows the stabilization of myosin with F-actin. In accordance with this information, conformational changes in myosin superfamily are regulated by rotation of the lever arm that affects their association with filamentous actin.[22]

The phylogeny of class I myosins suggest association of functions between myosins of vertebrates and invertebrates (Fig. 2). For example, the loss of MyoA in *Dictyostelium* brings a reduction in chemotaxis[23]; a similar phenomenon has been described for Myo1g in B cells.[24] Another example is MyoE from *Dictyostelium discoideum* that can bind to the same phospholipids (PIP_3) of the membrane than Myo1g and Myo1f from mice.[25,26] Similarly, MyoB, that belongs to the long tail class I myosins, share similarity with Myo1e and Myo1f. MyoB interacts with actin waves,[27] while Myo1e, interacts with NPFs (WASp and CARMIL).[28–30] However, for Myo1f, the association is unknown. In summary, the homology among class I myosin from invertebrates and from vertebrates suggests they may share similar functions.

Eight class I myosins are expressed in human and mice, encompassing six short-tailed (Myo1a, Myo1b, Myo1c, Myo1d, Myo1g, and Myo1h) and two long-tailed (Myo1e and Myo1f) myosins.[31] Both short-tailed and long-tailed myosins have a TH1 (tail homology) domain enriched with basic amino acids, thus permitting its interaction with the plasma membrane. Long-tailed myosins additionally have a TH2 domain that is rich in prolines and a TH3 domain corresponding to an SH3 domain (Fig. 3).[6]

The canonical phosphoinositide-binding PH domain (equivalent to TH1) shows the KXn(K/R)XR general sequence and is located between sheets β1 and β2 of the TH1 domain.[32–36] This motif varies between class I myosin members, which suggests differences with regard to the affinity for phospholipids and therefore for the specific localization and functions of each myosin.[36,37] Protein divergence is an important event during organism evolution, and molecular motors are an example of this. Cellular motors include three superfamilies: dynein, kinesin, and myosin.[38] Class I myosins may represent an ancestral myosin conserved from yeast to vertebrates. *Schizosaccharomyces pombe* (Myo1p), *Saccharomyces cerevisiae* (myo3p and myo5p), and *Dictyostelium discoideum* (MyoA, MyoE, MyoF, MyoB, MyoC, MyoD, and MyoH) have some paralogs of short and long tail myosins.[19] MyoA, MyoE, MyoF, contain a TH1 region similar to Myo1c and Myo1g.[31]

This review focuses on the class I myosins expressed in the leukocytes from vertebrates. However, it discusses a number of myosins

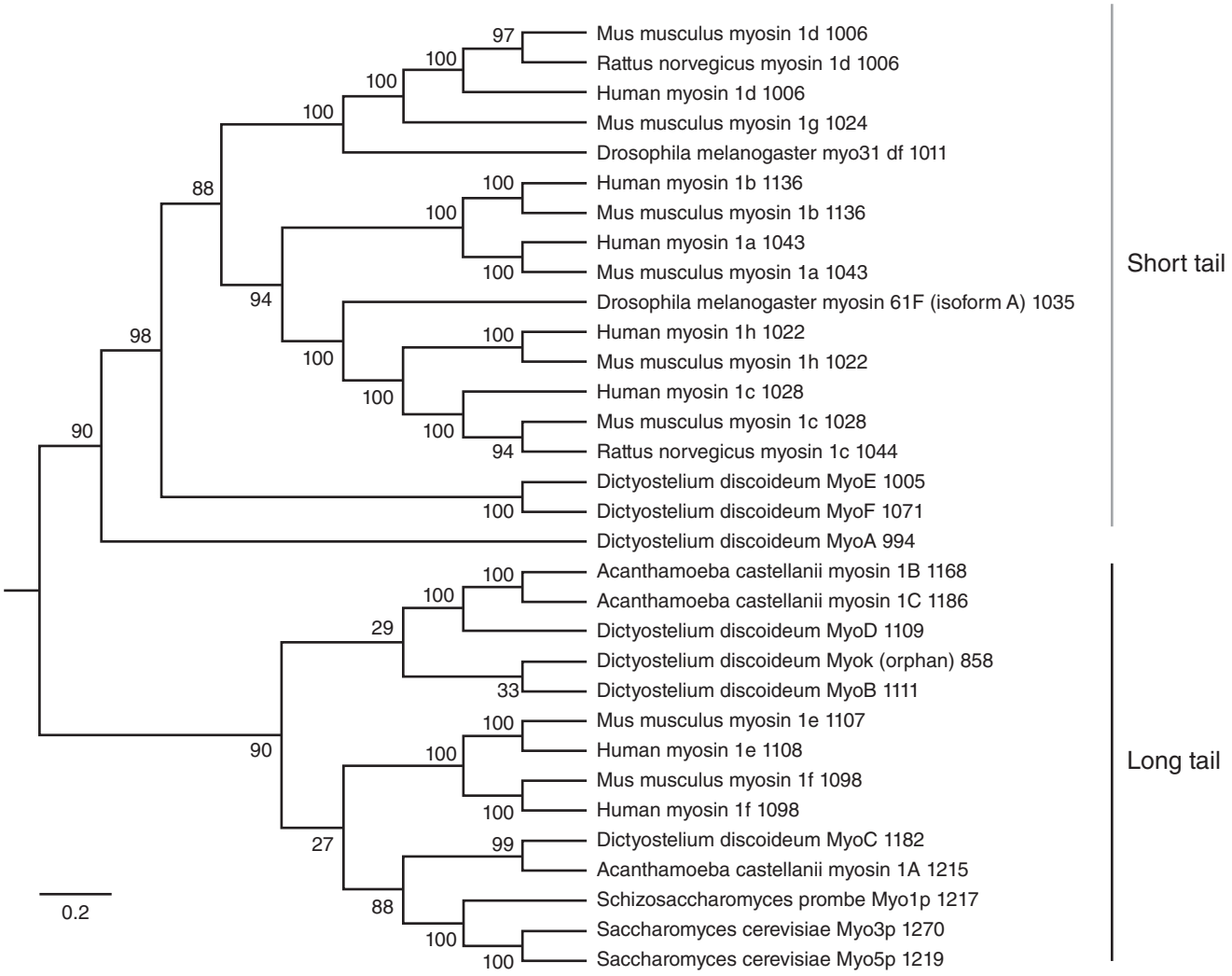

FIGURE 2 **Phylogenetic analysis of class I myosin.** The phylogenetic tree was constructed with the amino acid sequences from PubMed (Accession number): (NP_796364.2, NP_037115.2, NP_056009.1, NP_848534.2, NP_001188784.1, AAH53558.1, AAH54786.1, NP_005370.1, NP_001074688.1, NP_728594.2, NP_001094891.3, AAI44871.1, AAH44891.2, AAH21481.1, NP_037115.2, XP_636580.1, XP_636359.P22467.2, AAA27707.1, AAC98089.1, XP_643446.1, BAA76319.1, XP_636382.1, NP_851417.2, AAH98392.1, NP_444444.2, AAH28071.1, AAC98089.1, AAC35357.1, Q9Y7Z8.1, EIW09408.1, EWH16717.1) to build the phylogenetic tree, we used "MEGA 7" (https://www.megasoftware.net/). This analysis included 22 class I myosin of *Mus musculus*, *Rattus norvegicus*, *Homo sapiens*, *Drosophila melanogaster*, *Dictyostelium discoideum*, *Acanthamoeba castellani*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*. The scale bar denotes the number of substitutions per site

from invertebrates because they have suggested similar functions in vertebrates. From the eight class I myosins expressed in humans and mice, only three short-tailed myosins (Myo1c, Myo1d, and Myo1g) and two long-tailed myosins (Myo1e and Myo1f) have been described in leukocytes. Thus, this review only focuses on of these five class I myosins.

3 | REGULATION OF CLASS I MYOSINS

Class I myosins are regulated by Ca^{2+} concentrations, by the number of calmodulin-associated chains, and by phosphorylation.[39] Light chains, such as calmodulin or calmodulin-like proteins, are associated with the neck domain and have structural or regulatory functions (e.g., regulating the enzymatic activity of the head domain). Myo1c can bind three calmodulin molecules due to the presence of three IQ motifs.[40–42] However, regulation of these molecules in vertebrates is still a poorly explored field.

Myosin function regulation derives from the study of class II and V myosins in vertebrates and class I myosins in lower eukaryotes, such as *Dictyostelium* and *Acanthamoeba*, where the role of phosphorylation has been extensively described. The head region has different sites of phosphorylation that takes place in the TEDS region (defined as the region containing one of these amino acids: threonine (T), glutamate (E), aspartate (D), or serine (S). This region is found 16 amino acids upstream of the DALAK sequence). This sequence is conserved in most vertebrate and invertebrate myosin classes. Phosphorylation in the TEDS region produces a constitutive activation, increasing motor activity and leading myosin to localize in pseudopods and phagocytic cups,[43] Phosphorylation at these sites causes an increase in enzymatic activity. Mutations in TEDS residues involve a loss or

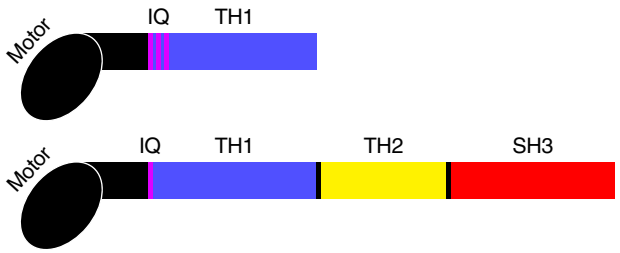

FIGURE 3 General structure of class I myosins. Class I myosins have a head domain (motor domain contains the actin binding site), a neck domain (contain IQ regions) and a tail domain (contains TH1, TH2 and TH3 regions, for binding to different molecules). These myosins are classified as short-tailed and long-tailed depending on the number of regions present in the tail

reduction in activity.[43] The class I myosins of invertebrates are phosphorylated mainly in serine, while in vertebrates this site is occupied by a negatively charged aspartate or glutamate suggesting that phosphorylation in the TEDS region is no longer required.[43] The phosphorylation in invertebrates is performed by the p21-activated kinase family[44] and in *Acanthamoeba*, this phosphorylation produces a rise of the actin-activated ATPase activity by rising the rate of phosphate release.[44] The phosphorylation are mainly observed during events such as motility and pseudopod or uropod formation.[45,46]

There are few reports of post-transcriptional modifications in class I myosins in vertebrates. For example, Wenzel and colleagues, through a phospho-proteome analysis of murine macrophages showed that myosin 1e and myo1f undergo threonine and serine phosphorylation in the tail region, in response to LPS. However, the consequences of this phosphorylation were not addressed in that work.[47] However, in class I myosin, the manner in which these modifications regulate myosin function is unknown, thus requiring further research.

4 | MYOSIN 1C: FROM MEMBRANE TO NUCLEUS; THE MOST VERSATILE CLASS I MYOSIN

Myosin 1c is a highly diverse and specialized protein having nuclear and cytoplasmic functions expressed in numerous tissues.[48] Recently, three isoforms were reported. These isoforms show several differences in N-terminal regions. These differences affect the specific nucleotide-binding properties of Myo1c isoforms, adding diversity to the kinetics of association with actin.[49] One isoform is present in the nucleus and the IQ sites are important for its translocation. Two out of its three IQ domains are needed for Myo1c nuclear transportation. The deletion of the first two IQ domains prevents its nuclear localization while its cytoplasmic retention is not affected. The third IQ domain does not participate in its nuclear localization or retention. A putative nuclear localizing sequence (NLS) is found within the first two IQ domains (residues 712–770). Interestingly, the second, but not the first IQ domain, contains a NLS sequence that promotes its nuclear accumulation. The traffic of Myo1c to the nucleus depends on importin 5, importin 7, and importin-β1 binding to an IQ complex. When calcium rises, calmodulin frees the site for the recognition by importin-β1, allowing Myo1c transportation into the nucleus.[50]

Within the nucleus, Myo1c engages in transcription, mRNA maturation and chromatin remodeling due to its interaction with RNA polymerases I and II[51–53] and with SNFH2, a molecule pertaining to the chromatin remodeling complex B-Wich.[54–56]

Within the cytoplasm, myosin 1c has a specific localization in actin-rich structures near the plasma membrane, where it is necessary for the membrane's fusion process, such as the autophagosome–lysosome fusion.[57] Also, this molecular motor is involved in the mobilization of vascular endothelial growth factor receptor-2 (VEFGR2) in response to VEGF,[58] as well as in recycling the transferrin receptor (TfR) and the traffic and exchange of cargo proteins to kinesin 1.[59] In HeLa cells, Myo1c is located in lipid rafts and participates in lipid raft mobilization, *Salmonella* invasion, cell spreading, and cell migration.[60] However, in immune cells, this myosin is located in dendrites (short-branched extension of the membrane produced by dendritic cells and activated B lymphocytes similar to the structures shown by neurons).[61] Furthermore, this protein is enriched with lipid rafts of B lymphocytes containing MHC-II. Thus, Myo1c regulates spreading and antigen-presenting capabilities.[61]

Myo1c is a potential autoimmune disease marker. The Ser-Pro-Cys sequence present in a 48 kDa Myo1c form is found at high concentrations in serum from multiple sclerosis and rheumatoid arthritis patients and absent in serum from healthy subjects.[62]

Myo1c has different functions in cell physiology as a result of its numerous interactions with other proteins, such as Glut-4, calmodulin-like (CIB1, CaBP1), and G-actin.[63–65] Although, Myo1c is a short-tailed myosin lacking protein interaction domains, its related light chains (e.g., calmodulin) might mediate new interactions. Thus, Myo1c can be referred to as the most versatile class I myosin member.

5 | MYOSIN 1D: IMPLICATIONS IN DENDRITIC-CELL ACTIVATION

The information on Myo1d is limited. Initial studies identified Myo1d expressed in the liver and involved in the development of the central nervous system.[66,67] However, recent reports suggest the participation of this myosin in immune cells. An increase in MYO1D gene expression induced by serotonin has been reported in alveolar macrophage.[68] Furthermore, Myo1d regulates CD86 and MHC-II expression in the plasma membrane of dendritic cells in response to neuraminidase (i.e., the structural protein of the influenza virus). Neuraminidase induces the upregulation of miR-155 and miR-674. MYO1D is a target gene of miR-155 and dendritic cells lose the ability to raise their activation molecules when MYO1D is silenced.[69] Thus, Myo1d might play an essential role in immune responses against viruses. Histone methyltransferase DOT1L regulates MYO1D expression during osteoclastogenesis. DOT1L inhibition induced an increase in Myo1d mRNA. Nonetheless, the mechanism used by DOT1L to regulate Myo1d is unclear.[70] In accordance, Myo1d is regulated by different mechanism but some functions still unexplored.

6 | MYOSIN 1E: REGULATES MHC-II TRANSPORTATION AND PHAGOCYTIC CUP FORMATION

Myo1e was initially identified in actin-rich areas near the plasma membrane,[71] subsequently reported by other authors.[72,73] Moreover, Myo1e is an important component of invadosome (actin-rich adhesion structures, important for degradation and invasion of extracellular matrix).[72] Studies on macrophages have found this myosin surrounds recently formed phagosomes.[74,75]

The Myo1e tail binds to large unilamellar vesicles containing anionic phospholipids, such as phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) or phosphatidylserine.[76] Myo1e combines with LSP1, a molecule that is involved in Fcγ-receptor-mediated phagocytosis in macrophages.[77] An additional exciting interaction is the connection established between Myo1e and ARF7EP during MHC-II-mobilization in human dendritic cells. ARF7EP exports MHC-II to the membrane; therefore, Myo1e could be involved in antigen presentation.[78]

The absence of Myo1e in LPS-stimulated macrophages reduces cell spreading, chemokine secretion, and antigen presentation as a result of the reduced MHC-II expression in the membrane.[47]

The tail domain expression of this myosin acts in a negative dominant manner to inhibit transferrin endocytosis,[28,79] suggesting the participation of Myo1e in this clathrin-mediated process. Myo1e is a member of a candidate group of genes which are a target of BTK. The absence of BTK reduces the expression of Myo1e.[80] The role of Myo1e in B cell maturation has not been explored.

The diversity in the functions, expression and localization of this motor protein could indicate its increased importance among class I myosins. Thus, further studies are needed to fully understand the multiple roles of this protein in different settings.

7 | MYOSIN 1F: CONTROLLING IMMUNE CELL ADHESION

Similar to Myo1e, Myo1f is expressed in immune cells and is enriched in F-actin areas.[81] In monocyte-derived macrophages, Myo1f regulates macrophage spreading after LPS stimulation[47] and is a component of tunneling nanotubes (TNT).[82] TNT are microstructures made up by actin filaments and microtubules to transport organelles, receptors, and RNA molecules.[82,83] Thus, Myo1f could be a motor protein involved in molecular trafficking within these cells. Moreover, dysregulation in MYO1F gene was reported in macrophages from patients with CD40L deficiency, before and after treatment with rhIFN-γ[84]; whereas, that in T cells has been described an increase of MYO1F gene after CD28 stimulation.[85] These observations suggest Myo1f is a myosin that responds to cellular activation and could be involved in specific functions after this stimulation.

In neutrophils, Myo1f controls the exocytosis of vesicles containing β2 integrins. Myo1f-deficient neutrophils show an increased expression on the surface of these adhesion molecules.[81] This phenomenon has implications in vivo, where the increased adhesion of neutrophils prevents their migration and consequently, the cells fail to control the *Listeria monocytogenes* infection.[81] These data suggest Myo1f could regulate the cortical tension generated by the actin filaments associated with the plasma membrane. However, this mechanism has not been demonstrated by the authors. This myosin was demonstrated to be essential for cell extravasation during inflammation, allowing the deformation of the nucleus as a necessary step during neutrophil migration.[86]

Our group recently reported that Myo1f plays an important role in intestinal inflammation. This protein is induced in colonic macrophages and its presence positively influenced the accumulation of αVβ3-integrin. This integrin enhances intercellular adhesion and stimulates a proinflammatory phenotype by inducing the activation of ILK/Akt/mTOR signaling which in turn induce STAT1 and STAT3 activation. Macrophages lacking Myo1f failed to fully commit into a M1-phenotype. Interestingly, we also observed that Myo1f upregulation leads to an enhanced secretion and production of IL-1β in macrophages, in vivo and in vitro. Consequently, in a model of colitis DSS-induced Myo1f-deficiency ameliorated epithelial cells damage and reduced disease symptoms while enhanced epithelial restitution.[87]

The MYO1F gene has been reported to be fused to the MLL gene in acute monocytic leukemia[88–90] or to VAV1 in peripheral T cell lymphomas.[91] However, to date there is no explanation as to how the MYO1F gene contributes to generating these diseases. In summary, Myo1f is a protein with different functions important in macrophages and neutrophils.

8 | MYOSIN 1G: REGULATION OF PLASMA MEMBRANE TENSION

Myo1g was first identified as a minor human histocompatibility antigen HA-2, involved in the establishment of bone marrow transplantation.[92,93] Two genetic variants known as Myo1g (V) and Myo1g (M) having different respective sequences (YIGEVLVSV or YIGEVLVSM) were described in the literature. Both variants are presented by HLA-A*0201, although the V/M change in the sequence has a minor effect in binding to the MHC, as well as in recognition by T cells.[92]

Myo1g is exclusively expressed in hematopoietic cells, where Myo1g expression levels vary between different lineages, being especially abundant in B and activated T cells.[94] The analysis of lymphocyte proteomic profiles by mass spectrometry indicated that Myo1g is the most abundant class I myosin expressed by T lymphocytes. It localizes in the plasma membrane and is particularly enriched at the cell-surface microvilli of T[95] and B lymphocytes.[24] Myo1g is also present in lipid rafts from B lymphocytes[96] and neutrophils.[97] Aguirre-Gamboa and colleagues suggest that Myo1g could be involved in the active regulation of B lymphocytes in humans. This assumption arises from the presence of a locus in chromosome 7, which is associated with the number of B cells in humans. In this locus, MYO1G is regulated by SNP rs10277809, which in turn affects the expression of Myo1g levels.[98]

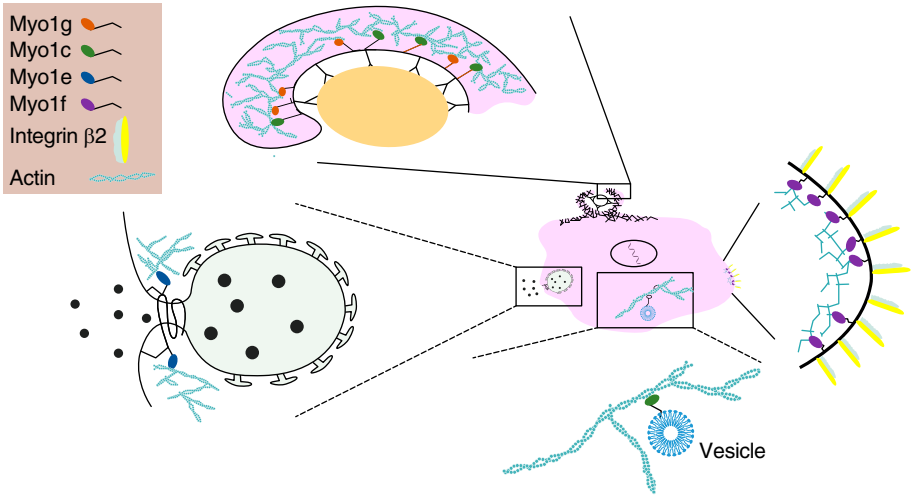

FIGURE 4 Functions of Class I myosins in immune cells. Class I myosins are monomeric proteins involved in actin polymerization, cell motility, cell shape control, sensory transduction, and vesicular trafficking. Specific functions include the maintenance of membrane projections, localization of integrins and molecules such as MHC class II, endocytosis, vesicular trafficking and membrane tension

Although there are very few reports concerning the role of Myo1g, it is known that this motor protein is involved in the maintenance and regulation of the plasma membrane tension in cells, especially in T[94] and B lymphocytes.[99] The reduction or the absence of Myo1g, using interference RNA or mice deficient in this protein, has shown that the elasticity of the membrane of T and B cells is reduced.[100]

Myo1g binds to phosphatidylinositol 3,4 bisphosphate (PtdIns(3,4)P2) and phosphatidylinositol 3,4,5 triphosphate (PtdIns(3,4,5)P3) in the plasma membrane.[101] These phospholipids are highly abundant in several endosomes[96,101,102]; where Myo1g is also found. Myo1g has been reported in endosomes of primary B lymphocytes and in the Raji cell line.[100]

Myo1g is also present in various types of exosomes, such as the exosomes secreted by PHA+IL-2 in T lymphocytes,[103] in the human thymus[100]; and in microvesicles of human neutrophils.[97]

However, Myo1g is also found in HIV-virions released by the THP-1 cell line[104] or the LPS-stimulated THP-1 cell line.[105] The presence of Myo1g in these membrane compartments suggests an essential role in a variety cell types; however, the precise function of Myo1g is still under investigation.

Data from our group describes Myo1g participation in recycling lipid rafts and CD44 in B lymphocytes. The absence of this protein delays CD44 production, "hijacking" CD44 and lipid rafts within the B lymphocyte. Myo1g, associating with RhoA GTPases, causes a reduction in the length and number of several membrane-protrusions in B lymphocytes.[96] Recently, Wang and colleagues described the decrease of Myo1g expression in the HL-60 cell line in response to arsenic trioxide.[106]

The abundance of Myo1g in activated T- and B-lymphocytes suggests the participation of this protein in several functions of these immune cells. However, mice deficient in this protein has not shown gross defects but subtle deficiencies, suggesting that other class I myosins may compensate its absence. Therefore, more studies are needed to increase our knowledge in this field.

9 | CONCLUDING REMARKS

In recent years, the conventional concept of myosins has seen some progress. Being formerly studied only in muscle cells, the analysis thereof has evolved to include many organisms, cells, and tissues. Myosins have become essential players in actin-cytoskeleton dynamics as well as in many diverse areas of cell biology, from gene regulation and proliferation to membrane tension and vesicular trafficking. The biophysical, biochemical, structural, and functional analyses of class I myosins have revealed numerous capabilities, advantages, restrictions, and disadvantages associated with this family of motor proteins (Fig. 4). From the eight members of this family, only Myo1c has been extensively explored. Consequently, further studies are needed to increase our knowledge in this field. These molecular motors have shown various expression patterns across cell populations, suggesting specific functions. Nevertheless, the absence of one of these members in some cases can be replaced for another family player, suggesting a number of redundancies in their functions. For all of these reasons, the study of this protein family poses both a challenge and an opportunity for any cell biologist having an interest in the functions performed by these motor proteins.

AUTHORSHIP

D.A.G-P., Z.L.P-Q, and L.S-A, outlined and structured the concepts covered in this review. D.A.G-P., Z.L.P-Q, and L.S-A, participated in writing, reviewing, and editing the manuscript. D.A.G-P., Z.L.P-Q, contributed equally in this manuscript. The final version of the manuscript was approved by L.S-A.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

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