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Ezrin-Radixin-Moesin family proteins in the regulation of B cell immune response

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Abstract

Dynamic reorganization of the cortical cytoskeleton is essential for numerous cellular processes including B and T cell activation and migration. The Ezrin, Radixin and Moesin (ERM) family proteins play structural and regulatory roles in the rearrangement of plasma membrane flexibility and protrusions through their reversible interaction with cortical actin filaments and plasma membrane. Recent studies demonstrate that ERM proteins are not only involved in cytoskeletal organization but also offer a platform for the transmission of signals in response to a variety of extracellular stimuli through their ability to crosslink transmembrane receptors with downstream signaling components. In this review, we summarize the present knowledge and recent progress made towards elucidating a novel role of ERM proteins in the regulation of B function in health and disease.

Keywords

Ezrin-Radixin-Moesin; B cell; membrane dynamics; microclusters; signaling; germinal center

I. INTRODUCTION

The generation of effective adaptive immune responses to pathogens necessitates the recognition of invading microbial pathogens by T and B cells, which display an extremely diverse repertoire of antigen-specific recognition receptors that enable specific identification and elimination of pathogens. While B cells can recognize the native protein antigens on pathogen surfaces, T cells do not directly recognize the surface molecules of pathogens. T cells specifically recognize the processed antigenic components of invading pathogens following presentation by antigen presenting cells (1, 2). Efficient accomplishment of these functions requires a finely regulated cellular cytoskeleton to enable reorganization of the cellular membrane, receptor localization, recruitment of signaling intermediates and changes in the morphology of the cell (3–7).

The Ezrin-Radixin-Moesin (ERM) family proteins are of particular interest in this regard as they play structural and regulatory roles in the assembly and stabilization of plasma membrane interactions through their ability to interact with transmembrane proteins and the

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cytoskeleton (8). In doing so, they provide structural links to strengthen the cell cortex and facilitate several key cellular process, including the membrane dynamics, substrate adhesion, cell survival, determination of cell shape, polarity, formation of membrane protrusions, cell adhesion and motility (9). The physiological function of ERM proteins is highly reliant on phosphorylation-dependent conformational changes, which modulate their membrane protein-actin cytoskeletal crosslinking capacity in response to growth factor, chemokine, and antigen stimulation (10–15). In addition to membrane-cytoskeletal remodeling, recent studies have shown that ERM proteins serve as intracellular scaffolds (16, 17), that facilitate signal transduction and consequently regulate B and T cell activation. In this review, we highlight the role of ERM proteins, focusing on ezrin in the regulation of membrane dynamics, and organization and control of molecular events during B cell immune response.

II. THE ERM FAMILY

A. A Historical perspective

The ERM family consists of three closely related proteins, ezrin, radixin, and moesin. Ezrin, the first ERM protein identified in many systems and the best studied member of this family, was originally isolated from chicken intestinal epithelial brush borders as a component of microvilli (18) in 1983 and named in recognition of Ezra Cornell, a founder of Cornell University. Ezrin is a protein of 585 amino acids with an isoelectric point of 6.15 and a theoretical molecular mass of 69 kDa. Radixin, a 583-amino acid polypeptide shares 75.3% identity with the human ezrin and 74.9% with the mouse ezrin (19), was isolated from rat hepatocyte cell junctions and found to localize to the cytoplasmic surface of adherens junctions in many cell types (20). Moesin was isolated from bovine uterus abundant in smooth muscle cells as a heparin-binding protein and called membrane-organizing extension spike protein (21). Moesin is a 577-amino acid polypeptide, which shares 71.7% sequence homology with the mouse ezrin (22).

Immunoblotting and immunofluorescence microscopy revealed that ERM proteins are co-expressed in most cultured cells but their expression appear to be regulated in cell type- and organ-specific manner (22–24). Moesin is the predominant isoform expressed in leukocytes, which generally express much lower levels of ezrin. Some leukocytes, such as B and T cells, do not express detectable levels of radixin, whereas other subtypes [e.g. natural killer (NK) cells and neutrophils] express trace amounts of radixin (25, 26). At the subcellular level, the three ERM are concentrated at cell-surface structures such as microvilli, filopodia, uropods, ruffling membranes, retraction fibers and cell adhesion sites where actin filaments are associated with plasma membranes (27, 28). However, the occurrence of ERM proteins in microvilli, particularly on the apical aspect of polarized cells, seems to be a common feature that implicates their contribution in the organization of this specialized plasma membrane domain.

B. Basic structural insights into ERM proteins

ERM proteins are highly conserved throughout evolution and it is likely that they have diverged from a common ancestral gene. Vertebrate genomes contain three genes encoding the three paralogs ezrin, radixin and moesin. On the contrary only one ERM gene is found in

the genome of lower eukaryotes (e.g. fruit flies and nematodes), suggest us that in vertebrates they probable ascended by gene duplication (9, 29). ERM proteins are characterized by a highly conserved N-terminal FERM (band Four point one Ezrin Radixin Moesin) domain also known as the N-terminal ERM association domain (N-ERMAD); a central α-helical domain (~200 a.a.) that is predicted to form coiled coils; and an actin-binding C-terminal domain (~100 a.a), also known as the C-terminal ERM-association domain (C-ERMAD) (Figure 1A). X-ray structure of the globular FERM domain revealed the presence of three subdomains (F1, F2 and F3; also known as A, B and C, respectively) that together form a compact cloverleaf structure. The subdomains have structural homology with ubiquitin, acyl-CoA-binding protein and the plekstrin-homology domain respectively (9). The co-crystal structure of the FERM domain with the $PI(4,5)P_2$ head group analog IP_3 revealed that the binding site is composed of positively charged cleft between the subdomains F1 and F3 (30). The C-terminal and α-helical domains can both fold back upon the FERM domain and mask both actin-binding and FERM domain interactions, leading to their inactive closed conformation. Regulation of this conformation by phosphorylation and phospholipid binding modulates ERM activity as discussed below.

C. Regulation of ERM protein activity

The first insights into the molecular mechanism of the regulation of ERM proteins came with the discovery that moesin is phosphorylated on T558 located in the C-ERMAD during platelet activation (31). Several other studies also demonstrated that phosphorylation of the similar threonine residue at T567 in ezrin and T564 in radixin can be considered a hallmark of activation. In addition, a two-step model of ERM activation has been suggested where the functional activation of ERM proteins is achieved through a sequential process involving phosphatidyl 4,5-bisphosphate (PIP_2) binding to a subdomain in the N-terminal FERM domain, plasma membrane localization, and threonine phosphorylation (Figure 1B) (32, 33). Inactive ERM proteins reside in the cytosolic fraction of cells, whereas activated ERMs are membrane-associated and bind with various integral membrane proteins by their N-terminal FERM domain and polymerized F-actin via the carboxyl-terminal domain.

Numerous kinases have been postulated to phosphorylate the C-terminus threonine residue of ERM proteins including myotonic dystrophy kinase-related Cdc42-binding kinase, G-protein-coupled receptor kinase 2, Rho-associated kinases, Nck-interacting kinase (10, 34–36). In lymphocytes a number of other kinase such as lymphocyte-oriented kinase and protein kinase C have been reported to activate ERM proteins by phosphorylation (37, 38). Ezrin can also be phosphorylated at T235 through cyclin-dependent kinase 5 and it has been demonstrated that mutation of T235 to aspartic acid enhances the membrane localization of ezrin (39). In addition to threonine residues, ezrin can undergo tyrosine phosphorylation upon stimulation by platelet-derived growth factor and hepatocyte growth factor (40). The major phosphorylated sites in these pathways have been mapped to Y145 in the N-terminal domain and Y353 in the α-helical region (40, 41). Phosphorylation of Y353, which is not conserved in other ERM proteins, is required for the binding of the regulatory p85 subunit of phosphatidylinositol 3-kinase (PI3K) to ezrin and for the activation of the PI3K protein kinase Akt signaling pathway in kidney epithelial cells (42). It has also been shown that the Src family kinase (SFK) Lck catalyzes phosphorylation of Y145 in Jurkat T-cells (43).

Furthermore, Heiska et al (44). have recently shown that Src-mediated phosphorylation of ezrin at Y477 is required for growth and invasion of Src-transformed fibroblasts in 3-dimenional (3D) matrix cultures. Although activation is the focus of studies of ERM protein regulation in most cells, ERM protein inactivation is also biologically important, particularly in cytoskeletal reorganization. Only myosin phosphatase has been clearly implicated in constitutive ERM dephosphorylation, while other phosphatases have been suggested, including PP2A or PP2C *in vitro* studies (45–47). In lymphocytes, reduction in PIP_2 levels that results from increased activation of PLCγ is sufficient to initiate ERM dephosphorylation and disassociation from cortical membrane (15).

D. Binding partners of ERMs

The C-terminal actin-binding domain of ERM proteins engages with the N-terminal FERM domain via “keystone interactions” to stabilize its folding and generate dormant monomers (48). In addition, ERM proteins can also exist as homo- and hetero-dimers wherein an intermolecular head to tail association stabilizes the two partners (49, 50). The cytosolic regions of certain integral membrane proteins may perform the same function in an active ERM protein. Indeed, the N-terminal FERM domains of ERM proteins were reported to directly bind to the cytoplasmic domains of CD44 and other transmembrane proteins such as ICAM-1, -2, and -3, L-selectin, P-selectin glycoprotein ligand-1 (PSGL-1), CD43 and CD95 (51–53). Like PIP_2, the calcium-binding EF-hand-like S100 protein was also shown to bind to the FERM domain in dormant ezrin and induce its partial activation (54). Activated ERM proteins can associate with the cytoplasmic tails of transmembrane proteins, either directly or via PDZ-domain containing scaffolding proteins such as Ezrin-binding phosphoprotein of 50 kDa (EBP50; also known as NHERF1) and NHE3 kinase A regulatory protein (E3KARP; also known as NHERF2) (55). ERM proteins utilize different regions within the FERM domains to interact with different binding partners, and could therefore potentially interact concurrently with EBP50 and/or more than one integral membrane protein. Indeed, EBP50 links lipid raft-resident Csk-binding protein (Cbp) with ERM in T cells, thereby anchoring lipid raft to the cytoskeleton, and plays a negative role in immune synapse formation (56). Although most membrane proteins seem to bind all three ERM proteins, the death receptor Fas/CD95 binds to ezrin, but not moesin, in T lymphocytes (53). Using mass spectrometry-based interactome analysis, we recently identified a novel interaction between ezrin and an unconventional myosin motor protein, Myo18aα (57) in B cells (58). This interaction is particularly attractive as Myo18aα was previously reported to regulate protein localization, trafficking, migration and organelle morphology in epithelial cells (59–61). The binding of ezrin to Myo18aα and their co-localization with BCR signalosomes (58) suggests that this complex may serve to control BCR transport and signaling in antigen-stimulated B cells. Given the potential for joint regulation of B cell function by ezrin and Myo18aα, it will be important to identify the minimal binding site(s) on each protein that participates in their association.

III. ERM FUNCTION IN B CELL PHYSIOLOGY

A. B cell migration

B cell migration is essential for their development, and entry into secondary lymphoid organs is a prerequisite for the development of an effective antigen-specific humoral immune response. Cell migration is a highly integrated multistep process that is initiated by the exchange of plasma membrane microvilli for filopodia and lamellipodia, and physical advancement of the leading edge of the cell (62). Formation of these structures is driven by spatially- and temporally-regulated membrane-cytoskeletal remodeling at the leading edge (63). There is considerable evidence that ERM proteins are required for the formation and maintenance of microvilli. Indeed, ezrin was initially isolated from chicken intestinal microvilli (23). Threonine phosphorylation of ERM proteins correlates with the formation or elongation of microvilli in both epithelial and lymphoid cells (36, 64). We have recently reported that conformational switching of ezrin is vital for the generation and maintenance of microvilli, cell morphology and migration in resting B cells upon stimulation with chemokine (12). High-resolution total internal reflection fluorescence (TIRF) microscopy of resting B cells demonstrated that ezrin and moesin were localized in the cortical region beneath the cell membrane and enriched in the plasma membrane microvilli. When B cells were stimulated with the chemokine, B lymphocyte chemokine (BLC) or stromal cell derived factor 1α (SDF-1α), the C-terminus threonine residue of both ezrin and moesin underwent dephosphorylation concomitant with rapid co-polarization of ezrin with F-actin (12). Exogenous expression of constitutively active phosphomimetic mutant of ezrin (T567D) that traps the protein in an open conformation, significantly inhibited chemokine-induced transwell migration of B cells. Furthermore, scanning electron microscopy of B cells stably expressing T567D mutant of ezrin caused collapse of microvilli and prevented chemokine-induced generation of membrane ruffles. In addition, inhibition of ERM dephosphorylation by the serine/threonine phosphatase inhibitor, calyculin A, prevented chemokine-induced chemotaxis of B cells, as well as impaired B cell homing to lymph nodes and spleen *in vivo*, confirming that dephosphorylation of ezrin is a necessary prerequisite for chemokine-induced changes in B cell morphology and migration (12). Since our data revealed co-polarization of endogenous F-actin and ezrin in response to chemokine, we hypothesized a potential role of ezrin at the leading edge of migrating B cells. Time-lapse fluorescence imaging of B cells expressing a yellow fluorescent protein (YFP) fusion construct of wild type ezrin (Ez-YFP) showed predominant localization of ezrin at the cortical region. A majority of the cells expressing YFP fusion construct of the T567D mutant of ezrin (TD-YFP) also showed cortical localization of TD-YFP; however, a smaller fraction of the cells exhibited a polarized localization of TD-YFP at the uropod (12). Cell adhesion molecules such as ICAMs, CD43 and CD44 localize to the uropod and ERM proteins are crucial for anchoring these cell adhesion molecules to the substratum (65). We observed that the adhesion molecule CD44 was strongly colocalized with TD-YFP in spontaneous uropods. Therefore, we suggest that CD44 polarization induced upon strong attachment to the substratum, can force passive polarization of TD-YFP. Following chemokine stimulation Ez-YFP relocated to the lamellipodia, whereas TD-YFP failed to do so (66), suggesting that active dephosphorylation and detachment from plasma membrane anchors is essential for relocation of ezrin to the leading edge. Collectively, our findings

indicate that ezrin modulates B cell migration in a two step process: the initial dephosphorylation of ezrin allows membrane remodeling and morphological changes, followed by its recruitment to the actin-rich lamellipodia thereby provides the force for onward movement of B cells. There is considerable evidence to indicate the involvement of ERM proteins and Rho family GTPases in establishing and maintaining leukocyte polarization. It would be very interesting to identify the molecular pathways that lead to chemokine-induced dephosphorylation of ezrin.

B. B cell receptor mobility and intracellular signaling: role of ERM phosphorylation

1. Threonine phosphorylation—In order to become activated to produce antibodies, B cells must first recognize antigen through the B cell receptor (BCR), which induces rapid reorganization of B cell cytoskeleton, leading to the initiation of intracellular signaling. The dynamic nature of ERM proteins provides a regulated linkage between integral membrane proteins and actin cytoskeleton, thereby building spatially distinct compartments, which limit lateral movement of BCR. We have demonstrated that ezrin is primarily present in an open active conformation in resting B cells and associates with lipid rafts through Csk-binding protein (Cbp) (14). In response to BCR stimulation ezrin undergoes rapid and transient dephosphorylation at the critical threonine residue (T567), which results in a closed structure that is incapable of binding to actin and lipid rafts, leading to lipid raft coalescence (Figure 2). The ectopic expression of a phosphomimetic mutant of ezrin (T567D) that constitutively tethers lipid rafts to the actin cytoskeleton interferes with BCR-induced raft coalescence.[14] By employing TIRF microscopy, Treanor *et al* (67) demonstrated that ezrin-mediated linkage of the plasma membrane protein to actin filament restricts steady-state BCR dynamics by creating diffusion boundaries. Interference with the ezrin- and actin-defined network using actin-depolymerizing agent latrunculin A also causes an increase in BCR diffusion and triggers BCR signaling (67). Similarly, expression of a dominant negative N-terminal truncated construct of ezrin (Ez-DN) that cannot bind to F-actin but can still bind to integral membrane proteins increases BCR diffusion, whereas overexpression of Ez-TD, which stabilizes the linkage between plasma membrane and actin, dramatically reduces BCR diffusion (67). Specific recognition of antigen by BCR triggers rapid spreading of the B cell over the antigen-associated membrane, thereby driving the formation of numerous antigen receptor microclusters, which act as sites of active signaling through the recruitment of intracellular signaling molecules and adaptors. It is well established that transient dephosphorylation of ERM proteins and cytoskeletal reorganization play an important role in orchestrating and coordinating molecular processes (6, 14, 68). The initial dephosphorylation of ERM proteins following BCR engagement increases BCR diffusion by collapsing the spatial confinement zones and enables antigen receptor microclustering, which is ultimately stabilized due to rephosphorylation of ERM proteins and repolymerization of actin at later times (14, 67, 69). Disruption of ERM function by expression of Ez-DN was also shown to result in the formation of fewer antigen microclusters when stimulated on planar lipid bilayer containing antigen. On the contrary, overexpression of constitutive active mutant of ezrin (Ez-TD) leads to larger but reduced number of antigen aggregates (69). Furthermore, B cells expressing either Ez-DN or Ez-TD show reduced ERK phosphorylation following antigen engagement (69). These findings confirm that phosphorylation-dephosphorylation of ezrin at the threonine residue (T567)

offers an elegant mechanism to dynamically regulate the linkage between plasma membrane and actin filaments to adjust BCR diffusion dynamics, BCR clustering and downstream signaling. Thus, disruption of ezrin's function affects both lipid raft coalescence and membrane-cytoskeletal compartmentalization of the BCR molecules, suggesting the following model. Antigen stimulation leads to transient dephosphorylation of ezrin, which simultaneously causes breakdown of plasma membrane-actin cytoskeletal boundaries, BCR clustering, association of BCRs with lipid rafts, and lipid raft coalescence (Figure 2). These events unite the BCRs with lipid raft-resident Src family kinases for initiation of tyrosine phosphorylation. Continuous antigen stimulation causes rephosphoryation of ezrin, reinstatement of the membrane-cytoskeletal connections, which traps and immobilizes the raft-localized BCR clusters and signal transduction (Figure 2).

2. Tyrosine phosphorylation—In addition to the conserved threonine residue, ezrin undergoes phosphorylation at a unique tyrosine 353 (Y353) in B cells. Phosphorylation of ezrin on Y353 was first documented in human B cells after CD81 crosslinking. Engagement of CD81, a member of the tetraspanin family of proteins, induces spleen tyrosine kinase (Syk) to phosphorylate ezrin at Y353 (70). CD81 interacts with ezrin resulting in the recruitment of F-actin to membrane, thereby facilitates cytoskeletal reorganization and cell signaling. In this context, it is interesting that ezrin also contains two tyrosines in an ITAM-like configuration that becomes phosphorylated during the course of B cell activation (71). Moreover, we have recently described a role for antigen-induced phosphorylation of ezrin at Y353 in regulating the strength of BCR-dependent c-Jun N-terminal kinase (JNK signaling) (17). BCR crosslinking induces Syk-dependent phosphorylation of ezrin at Y353 that co-localizes and co-traffics with the BCR through early and late endosomes. Expression of a non-phosphorylatable Y353F mutant of ezrin exhibits decreased co-localization with the endocytosed BCR. BCR stimulation also promotes the association of tyrosine-phosphorylated ezrin with JNK and its upstream kinase MAPKK7 as well as spatial co-localization with phosphorylated JNK in the endosomes (17). However, expression of Y353F mutant of ezrin blocks antigen-induced JNK activation. These data clearly indicate that tyrosine phosphorylated-ezrin plays a crucial role in BCR signaling by acting as an adaptor to spatially localize the JNK signaling module in the proximity of the BCR signalosome (17). Since JNK activation is associated with increased cell survival, it is tempting to speculate that B cell survival may be regulated by tyrosine phosphorylation of ezrin.

C. B cell immune response in conditional knockout mice

So far, the role of ERM proteins in B cell function has been investigated by expressing different mutant constructs of ezrin or with siRNA-based gene-silencing approaches in cell lines. One of the central issues facing biomedical research is the need to translate *in vitro* data obtained in cell lines into knowledge about protein function in mice or human. Importantly transient knockdown has numerous disadvantages, such as variability, incompleteness of knockdown, potential non-specificity of reagents and sometimes it is challenging to entirely understand the true endogenous function of the molecule *in vivo*. To evade these limitations we generated a mouse line with conditional deletion of ezrin exclusively in the B cell lineage (72).

1. Role of ezrin in B cell homeostasis—To investigate the role of ezrin in B cell development and homeostasis, we analyzed B cell subpopulations in the bone marrow and secondary lymphoid organs (spleen and lymph nodes) of wild type (WT) and Ezrin-deficient (Ez-def) mice. B cell development in the bone marrow was comparable between both genotypes, with similar numbers of pro-B cells, pre-B cells and immature B cells (72). The numbers of mature circulating IgM^+CD19^+ B cells in the spleen, lymph nodes and bone marrow were also unaffected by deficiency of ezrin. Total numbers of follicular, marginal zone and B1 mature subsets in the spleen were equivalent in Ez-def and WT mice (72). In contrast, the number and proportion of $CD19^+CD5^+CD1d^{hi}$ regulatory B cells, recently described as a major B cell subset responsible for producing IL-10 (B10) (73, 74) in the spleen were substantially increased by lack of ezrin (our unpublished data). Taken together, these data showed that ezrin is dispensable for overall B cell development and homeostasis, however its absence leads to increased spontaneous B cell differentiation into the B10 subtype.

2. Role of ezrin in the initiation of B cell receptor signaling—As various mutants of ezrin have been reported to alter BCR microclustering (69), we employed TIRF microscopy to examine the effect of ezrin deficiency on this phenomenon in response to antigen in both naïve and LPS-primed B cells from WT and Ez-def mice. By using stochastic optical reconstruction microscopy (STORM) we confirmed an increase in size and intensity of BCR microclusters in the absence of ezrin, indicating that each cluster contains more BCR molecules. The steady-state BCR diffusion coefficient was unaffected by the loss of ezrin, whereas BCRs displayed a significantly reduced diffusion constant in antigen-induced Ez-def B cells (0.017 $\mu m^2/s$) compared to WT cells (0.034 $\mu m^2/s$) (75). The growth and aggregation of BCR microclusters coincided with reduced lateral mobility of BCRs in the two dimensional fluid plane of the plasma membrane. These observations led us to the conclusion that BCR confinement may be ineffective in the absence of ezrin, and that ezrin-based corrals serve to prevent uncontrolled increase in cluster size. It is well known that cross-linked B cell receptor (BCR) aggregates on the cell surface and finally assembles into the “cap” (76). Consistent with slower mobility, the BCR clusters exhibited delayed capping in the absence of ezrin. We further examined the impact of ezrin deficiency on raft dynamics by using fluorescent cholera toxin B. In comparison to WT B cells, Ez-def cells showed accelerated antigen-induced lipid raft coalescence (72). Single particle tracking studies have demonstrated that crosslinking of BCRs by antigen induces their association with lipid rafts, which leads to rapid recruitment of complex signaling assemblies that contain co-receptors for B cell activation as well as key enzymes and adaptor molecules (76, 77). The lower velocity and diffusion constant of BCR clusters in the absence of ezrin indicated that they support stronger signaling. As expected, the bigger BCR clusters formed in the Ez-def B cells were associated with more tyrosine-phosphorylated proteins. Immunoblotting analysis of Ez-def B cells showed an increase in the intensity of multiple tyrosine-phosphorylated protein bands, demonstrating a global amplification of the proximal BCR signaling module (75). Consistent with this, loss of ezrin in B cells led to increased activation of proximal signaling molecules, including Igα, Syk and PLCγ. In addition, activation of the signaling mediator ERK was robustly augmented in the absence of ezrin, whereas JNK activation was reduced (75). These findings are also consistent with the model presented in Figure 2, which

proposes that ezrin serves to limit antigen-induced BCR clustering and signaling, and lipid raft aggregration. Accordingly, loss of ezrin enhances all of these features of BCR activation.

3. Role of ezrin in humoral immune response—The engagement of specific antigen by the BCR leads to formation of numerous BCR-antigen clusters, which recruit a cascade of intracellular signaling leading to B cell proliferation and differentiation into cells able to produce protective antibodies (78). The robust anti-IgM-induced BCR signaling observed in Ez-def B cells correlated with increased proliferation and differentiation into antibody secreting cells (72). Moreover, Ez-def mice exhibited stronger antigen-specific antibody production *in vivo*. These observations demonstrate that ezrin tunes the strength of BCR signaling by regulating the size and dynamics of BCR microclusters. Interestingly, naïve Ez-def B cells displayed higher MHC II expression than the WT B cells suggesting that Ez-def B cells may engage in more efficient interactions with T cells, and enhance germinal center (GC) reactions and antibody affinity maturation. In this context it will be interesting to examine if and how ezrin deficiency in B cells controls the formation and function of GCs. Additionally, as autoimmune and malignant B cells exhibit dysregulated BCR signaling, further exploration of ezrin will also be clinically relevant in considering B cell therapeutics targeting BCR signaling. Furthermore, Ez-def mice offer a novel tool to examine the significance of membrane-cytoskeletal remodeling in B cell immunity, tolerance, autoimmunity and malignancy.

IV. ERM PROTEINS IN GERMINAL CENTER BIOLOGY

Protective immunity against pathogens depends on the production of high-affinity antibodies by long-lived plasma cells (PCs), which are generated from germinal centers (79). The GC is a highly dynamic microenvironment where highly specific antigen-activated B cells are selected to rapidly expand and differentiate into plasma cells to produce high affinity antibodies. It is therefore important to understand the biology of the germinal center. In GCs, B cells undergo antigen-dependent proliferation (known as centroblasts) in the dark zone (Figure 3) (80). During proliferation, B cells accumulate mutations at high frequency within the immunoglobulin heavy and light chain variable (V) region genes by a process termed somatic hypermutation (SHM) (81, 82). Centroblasts then differentiate into centrocytes and migrate to the light zone, where they are subjected to selection by T follicular helper cells (T_{FH}) in the presence of follicular dendritic cells (FDCs) (80). Selection is followed by terminal differentiation into memory B cells and plasma cells (Figure 3), which generate high-affinity antibodies that play a crucial role in clearing invading pathogens. Although, these molecular events are important for normal immune response, dysregulation in the germinal center program can lead to B cell disorders such as autoimmunity and lymphoma (83–86). Therefore, the molecular mechanisms and cell biology of the GC reaction may be possible targets for selective B cell therapy.

As the GC reaction is tightly regulated by communication between GC B cells and T_{FH} cells (Figure 3) (87), deregulated interactions can generate autoreactive B cells, which produce pathogenic autoantibodies (88). Currently, very little is known about the role of ERM proteins in autoimmunity. In a recent study by Li et al. (89), it was demonstrated that T

lymphocytes from blood of systemic lupus erythematous (SLE) patients exhibit increased phosphorylation of ERM proteins and higher expression levels of CD44. Immunofluorescence imaging showed that phosphorylated ERM and CD44 are highly expressed in T cells infiltrating in the kidneys of patients with lupus nephritis (89), suggesting a vital role of ERM proteins in the pathogenesis of the disease. However, nothing is known about ERM function in B cells in the context of autoimmune diseases. Given the crucial role of ERM proteins in B and T cell migration and modulation of intracellular signaling (12, 75, 90–92), it is possible that they may play essential role in mobility of B and T cells, interaction between them, providing survival signals and retention within the GC microenvironment. Interestingly, studies have shown that sphingosine-1-phosphate receptor 2 (S1PR2) is highly expressed in GC B cells (93) and T_{FH} cells (94). S1PR2 has been shown to induce phosphorylation of ERM proteins and formation of filopodia,[95] indicating a potential mechanism of cell migration within GC and needs to be explored in future. In addition, the survival of GC B cells depends on expression of ICAM1 on GC B cells, and lymphocyte-associated antigen 1 (LFA1) and bound antigen on FDCs. Furthermore, antigen presentation by GC B cells to T_{FH} cells also influences the survival of these cells through secretion of cytokines (96). ERM proteins have been shown to bind with cytoplasmic tail of ICAMs during cell migration and formation of immunological synapse (13, 97). Moreover, ERM proteins also orchestrate B and T cell activation as well as production of regulatory cytokines through their interaction with intracellular signaling modules (75, 91, 98). Therefore, it is conceivable that ERM proteins maintain the GC microenvironment, and dysregulation of their function may result in GC pathogenesis.

During the GC reaction, B cell DNA is modified through somatic hypermutation and class-switch recombination, which alter the B-cell receptor but at the same time may cause DNA damage that can ultimately lead to generation of different types of lymphomas such as Burkitt's lymphoma, diffuse large B cell lymphoma and follicular lymphoma (85). In a recent study it was shown that mutation in S1PR2 gene is associated with germinal center B cell (GCB)-derived lymphoma and deficiency of Gα13 promotes GC B cell survival and lymphomagenesis (99). As Gα13 regulates actin cytoskeletal remodeling (100), ERM-mediated membrane-cytoskeletal remodeling may also be a target in GCB lymphoma pathogenesis. Furthermore, Tzankov et al. (101) have demonstrated the expression of CD44 variant isoform 6 (CD44v6) in the primary tumor tissues from patients with diffuse large B cell lymphoma. CD44 is a type I transmembrane protein that is responsible for mediating communication and adhesion between adjacent cells and between cells and the extracellular matrix (102). CD44 variants are associated with tumor invasion and metastasis, and their expression is commonly allied with an unfavorable prognosis (102). Because activated ERM proteins bind to the cytoplasmic tail of CD44 through the FERM domain, it is tempting to speculate that activated ERMs and CD44 maintain the pathophysiology of GC-derived lymphomas by establishing a signaling network that governs B cell migration within the GC (Figure 3). ERM proteins are known to concentrate in and regulate the structure of cell surface projections such as microvilli, which are in turn important for nutrient adsorption, secretion and cellular adhesion (23). Hence, it is possible that ERM-mediated membrane structures regulate tumor microenvironment by providing nutrient/information exchange between cells, as well as secretion of cell survival factors such as cytokines and growth

factors. In fact, a high circulating level of vascular endothelial growth factor A (VEGF-A) has been detected in patients with non-Hodgkin's lymphoma, and tumor cells were identified as the producers of this growth factor *in vivo* (103). VEGF-A promotes tumor growth by stimulating tumor stromal cells to secret various cytokines including IL-4, IL-6 and IL-10, leading to paracrine activation of tumor cells (103), as well as stimulates cancer cell metastasis by upregulation and activation of ERM family proteins moesin through RhoA/ROCK-2 pathway (104). VEGF is also important for angiogenesis, and hence accelerates metastasis of tumor cells to lymph nodes and distant organs (105). Collectively, these studies suggest that ERM proteins may play important roles at multiple stages in modulation of the tumor microenvironment and their inhibition may offer a novel strategy to improve therapy against B cell malignancies.

V. CONCLUDING REMARKS AND FUTURE DIRECTIONS

Many recent breakthroughs, such as those that relate ERMs dynamics and B and T cell activation in real-time, were made possible by employing state-of-the-art approaches such as high-resolution TIRF and STORM imaging. Now it is well established that ERM proteins organize the interface between the actin cytoskeleton and the plasma membrane, and consequently play central role in determining the structure and function of plasma membrane and associated molecules. Indeed, ERM proteins regulate B and T cell activation through controlling BCR and TCR dynamics, scaffolding protein assembly and hence membrane associated intracellular signaling. In view of recent advancements in understanding the complex role of ERMs in lymphocyte functions, many fascinating questions remain to be countered. For example, to better grasp the molecular mechanism of ERMs regulation, further studies are necessary to identify the kinase(s) and phosphatase(s) that are responsible for phospho-cycling of ERM proteins. The ability of ERMs to act as cytoplasmic signaling scaffolds provides the potential for considerable functional diversity. Thus, further clarification of ERM binding partners and the contexts in which they interact, are an attractive area for future research. Furthermore, the physiological significance of tyrosine phosphorylation, including Y145 and Y353, in B- and T cell functions needs to be addressed. Since ERM proteins regulate both B and T cell activation and migration, it will be important to elucidate how these functions are affected in disease processes involving dysregulated lymphocyte activity.

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Abbreviations

ERM	Ezrin-Radixin-Moesin
BCR	B cell receptor
ICAM	intracellular adhesion molecule
PSGL	P-selectin glycoprotein ligand

MHC	major histocompatibility complex
GC	germinal center
T_{FH}	follicular helper T cell
FDC	follicular dendritic cell
S1PR2	sphingosine-1-phosphate receptor 2
TIRF	total internal reflection fluorescence
VEGF	vascular endothelial growth factor

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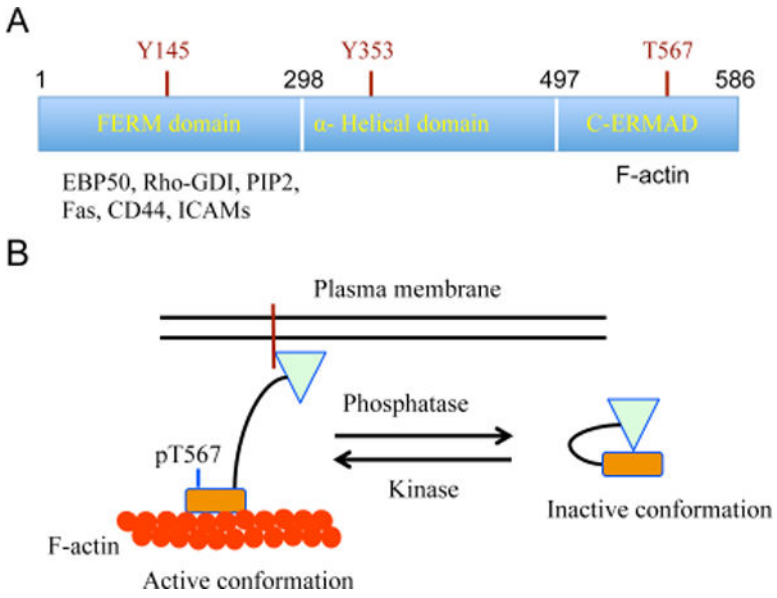

Figure 1. Domain organization of ERM proteins and a model for their regulation

A. The three ERM proteins exhibit a highly homologous domain structure. They consist of an amino-terminal FERM domain, which interacts with various transmembrane proteins, phospholipids and signaling molecules, followed by a helical domain and the C-terminal actin-binding domain. The F-actin binding site is positioned within the last 34 amino acid residues. The Y145 and Y353 residues are unique to ezrin and not conserved with radixin moesin. **B.** ERM proteins exist in equilibrium between an active "open" form and an auto-inhibited "closed" conformation. ERMs are activated by binding to phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphorylation at T567, which unmasks binding sites for F-actin and the cytoplasmic tails of specific membrane proteins. In the inactive state, actin and membrane binding sites are masked by intramolecular interaction of the N- and C-terminal domains.

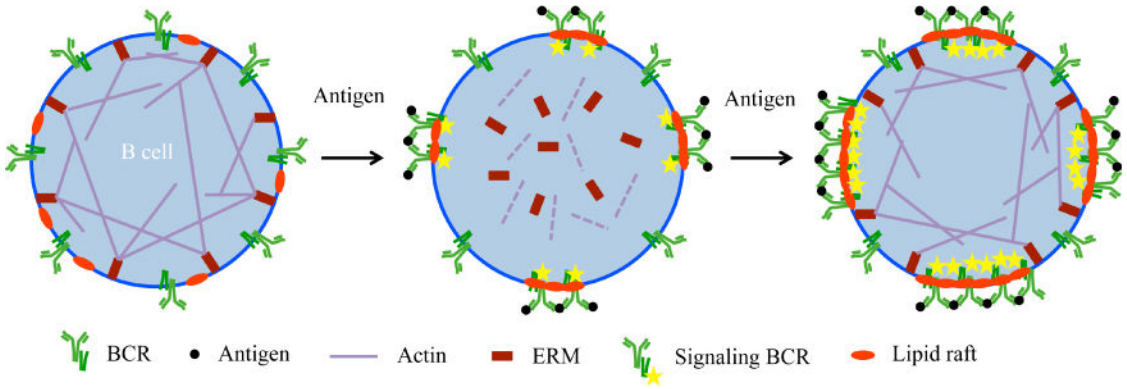

Figure 2. ERM proteins regulate B cell membrane raft dynamics and BCR clustering

In resting B cells, lipid rafts and BCRs are scattered randomly on the membrane, and have restricted lateral mobility within the membrane compartments created by activated ERM proteins and F-actin network. BCR engagement by antigen induces transient dephosphorylating of ERM proteins and their detachment from membrane protein and actin cytoskeleton. This molecular event enables the BCRs to form microclusters and associate with lipid rafts, and facilitates coalescence of individual membrane rafts into bigger entities, thus uniting the BCR clusters with proximal signaling kinases. Re-phosphorylation of ERM proteins upon continuous stimulation with antigen leads re-creation of membrane compartments in which the BCRs are immobilized and transduce signals.

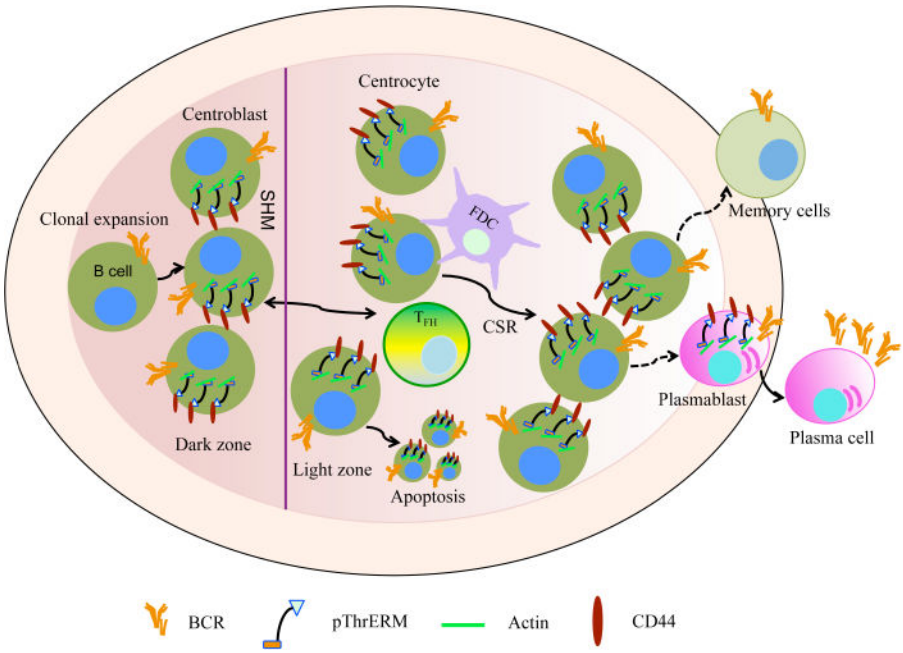

Figure 3. The germinal center microenvironment and potential role of ERM proteins in its regulation

Mature B cells are activated when they encounter cognate antigen. Activated B cells proliferate and differentiate into centroblasts in the dark zone of the germinal center. Dividing centroblasts activate the process of somatic hypermutation (SHM) in their immunoglobulin variable-region genes, leading to change in amino acid sequence and generation of antibody variants. Centroblasts then differentiate into resting centrocytes in the light zone and are selected for high affinity of their BCR for cognate antigen with the help of T follicular helper (T_{FH}) cells and follicular dendritic cells (FDCs). The centrocytes that are not selected undergo apoptosis and are removed. The immunoglobulin genes of a subset of centrocytes undergo class-switching recombination (CSR). Finally, selected germinal center B cells differentiate into memory B cells or antibody-secreting plasma cells. During the GC reaction ERM proteins may undergo higher phosphorylated as compared to naïve circulating B cells. The cycling of centroblasts and centrocytes between dark and light zone or exit from the germinal center may be tightly regulated by ERM proteins and cell adhesion molecules such as CD44.