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Unique ζ-chain motifs mediate a direct TCR-actin linkage critical for immunological synapse formation and T-cell activation

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TCR-mediated activation induces receptor microclusters that evolve to a defined immune synapse (IS). Many studies showed that actin polymerization and remodeling, which create a scaffold critical to IS formation and stabilization, are TCR mediated. However, the mechanisms controlling simultaneous TCR and actin dynamic rearrangement in the IS are yet not fully understood. Herein, we identify two novel TCR ζ-chain motifs, mediating the TCR's direct interaction with actin and inducing actin bundling. While T cells expressing the ζ-chain mutated in these motifs lack cytoskeleton (actin) associated (cska)-TCRs, they express normal levels of non-cska and surface TCRs as cells expressing wild-type ζ-chain. However, such mutant cells are unable to display activation-dependent TCR clustering, IS formation, expression of CD25/CD69 activation markers, or produce/secrete cytokine, effects also seen in the corresponding APCs. We are the first to show a direct TCR-actin linkage, providing the missing gap linking between TCR-mediated Ag recognition, specific cytoskeleton orientation toward the T-cell–APC interacting pole and long-lived IS maintenance.

Keywords: Actin microfilaments · Immunological synapse · T-cell activation · TCR

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Introduction

Upon TCR-mediated Ag-MHC recognition, polarized reorganization of TCRs together with additional cell surface receptors and intracellular signaling molecules is initiated toward the T-cell–antigen-presenting cell (APC) interface, segregating into receptor microclusters and eventually to a defined immune synapse (IS) [1–3]. The exact mechanism that controls the dynamics TCR rearrangement in the IS is as yet unknown. However, it is well established that TCR-mediated signaling controls synapse formation, since disruption of TCR signaling molecules such as LCK and VAV prevents this process [4, 5]. In addition, many studies have indicated that polymerization and remodeling of the actin-based cytoskeleton creates a scaffold critical to IS formation and stabilization [6]. Actin reorganization at the IS also plays a role in advanced stages of activation, enabling directed secretion of

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cytokines and execution of T-cell effector functions [7]. Disruption of the actin-based cytoskeleton or deficiency in key actin-regulatory proteins causes severe alterations of TCR-mediated activation progression [7].

Various studies including ours demonstrated that ~30% of the total TCRs are found in the detergent-insoluble cell fraction (dicf)-TCRs and were suggested as being linked to actin-based cytoskeleton via ζ. dicf-TCRs were shown to be expressed on the cell surface of both nonactivated and activated T cells [8, 9]. Although the unique features of dicf-TCRs, such as conformation and phosphorylation pattern [10] suggest a distinct role in T-cell function compared with that of detergent-soluble cell fraction (dscf)-TCRs, the mode of association with the cytoskeleton and their functional significance remain unclear.

It was previously published that upon TCR-mediated activation, although the majority of the receptors are internalized and degraded within 1–4 h, T-cell–APC interactions and TCR-mediated signaling are still evident for up to 10 h, and cytokine secretion persists for even longer (10–24 h) [11]. These findings raised several key questions as: what dictates the polarization of actin microfilaments toward the T-cell–APC interacting pole and IS formation, and what is the molecular basis for the specificity and stability of such a prolonged T-cell–APC interactions?

The cumulative data in the literature showing a significant role of the cytoskeleton in T-cell activation processes, led us to suggest that in nonactivated T cells a unique TCR-actin-based cytoskeleton linkage, most likely via the dicf-TCRs, could serve as the initiator for polar cytoskeleton remodeling upon activation. This could lead to the establishment of a signaling network toward IS formation, ensuing in the execution of full T-cell activation.

In the current study, we focused on the dicf-TCRs and discovered that these receptors are directly linked to actin via two positively charged motifs positioned within the ζ intracytoplasmic (IC) region and termed these receptors as cytoskeleton-associated (cska)-TCRs. We provide novel data showing the key role of the cska-TCRs in the execution of TCR-mediated activation processes leading to TCR clustering and a long-term signaling cascade resulting in cytokine synthesis and secretion. We summarize the studies in a model, illustrating the indispensable role of cska-TCRs in the prolonged IS maintenance and optimal T-cell and APC activation.

Results

Putative actin-binding motifs are localized within the ζ chain

Previous studies showed that TCR localization in the dicf depends on ζ [10] and that ζ could be coprecipitated with actin [9]. However, in neither the mode of interaction, whether it is direct or indirect, nor the molecular basis for this association and its functional significance were determined. We hypothesized that the dicf-TCRs could be major players in TCR-mediated polar actin filament polymerization toward the APC, leading to IS formation and T-cell activation.

To assess our hypothesis, we first examined whether ζ possesses regions that mediate its localization to the dicf. To this end, we tested the ability of different truncated ζ chains expressed in T-cell lines [12] and splenocytes from transgenic mice [13] (Fig. 1A) to localize to the dicf. The only truncation that abolished dicf ζ localization was the ζ-D66-150, which deleted a major part of the ζ IC region (Fig. 1B). This result was surprising since the CT-108 or the ζ-D66-114 truncations, which are complimentary, affected ζ-chain-dicf localization only slightly. Therefore, we raised the possibility that more than one ζ region might be responsible for mediating its dicf localization, whereby only the elimination of both, as in the ζ-D66-150, prevents this unique feature. Previous data showing ζ co-immunoprecipitated with actin in activated T cells [9] and that treatment with actin depolymerizing agents abolished dicf ζ localization [8] suggest that ζ might directly or indirectly interact with actin. A computer search revealed that ζ does not possess any of the previously described actin-binding motifs [14]. However, we discovered two RRR basic residue clusters within the mouse ζ, positioned at amino acids 102–104 and the other at amino acid 132–134 (Supporting Information Fig. 1). Positively charged residues were described for some proteins as mediating their association with F-actin [15, 16]. These ζ clusters are evolutionarily conserved (Supporting Information Fig. 1B), supporting their functional significance. Moreover, none of the remaining TCR subunits expresses these motifs.

Basic motifs within ζ mediate its direct association with F-actin and induce actin bundling

To test whether the basic residue clusters are important for ζ dicf localization and to identify which of the motifs is the most critical for this characteristics, we expressed in COS cells single mutated ζ molecules, changing the first RRR cluster to GGG (Proximal) or the second RRR motif to QQQ (Distal), or generated a double mutated molecule (MUT; Supporting information Fig. 1C). The results revealed that while each single mutation only partially disrupted dicf ζ localization, the double mutation almost completely abolished this localization as indicated by the dsfc/dicf ratios (Fig. 1C and Supporting Information Fig. 2). The residual minute dicf ζ found in the cells transfected with the double mutant molecule could be due to an incomplete lysis or some remaining dscf TCRs. These results suggested that ζ dicf localization could be conferred by its ability to directly bind actin and that a T-cell milieu is not required to support this linkage. Since the double mutation dramatically diminished dicf ζ localization within COS cells, we further proceeded our studies focusing on the double MUT.

We next assessed the capacity of in vitro-expressed ζ wild type (WT) or (MUT) IC domains to bind actin by using a cosedimentation assay. To this end fresh actin was polymerized in the presence of different concentrations of WT or MUT-fusion proteins, and the results revealed that only the WT ζ could be

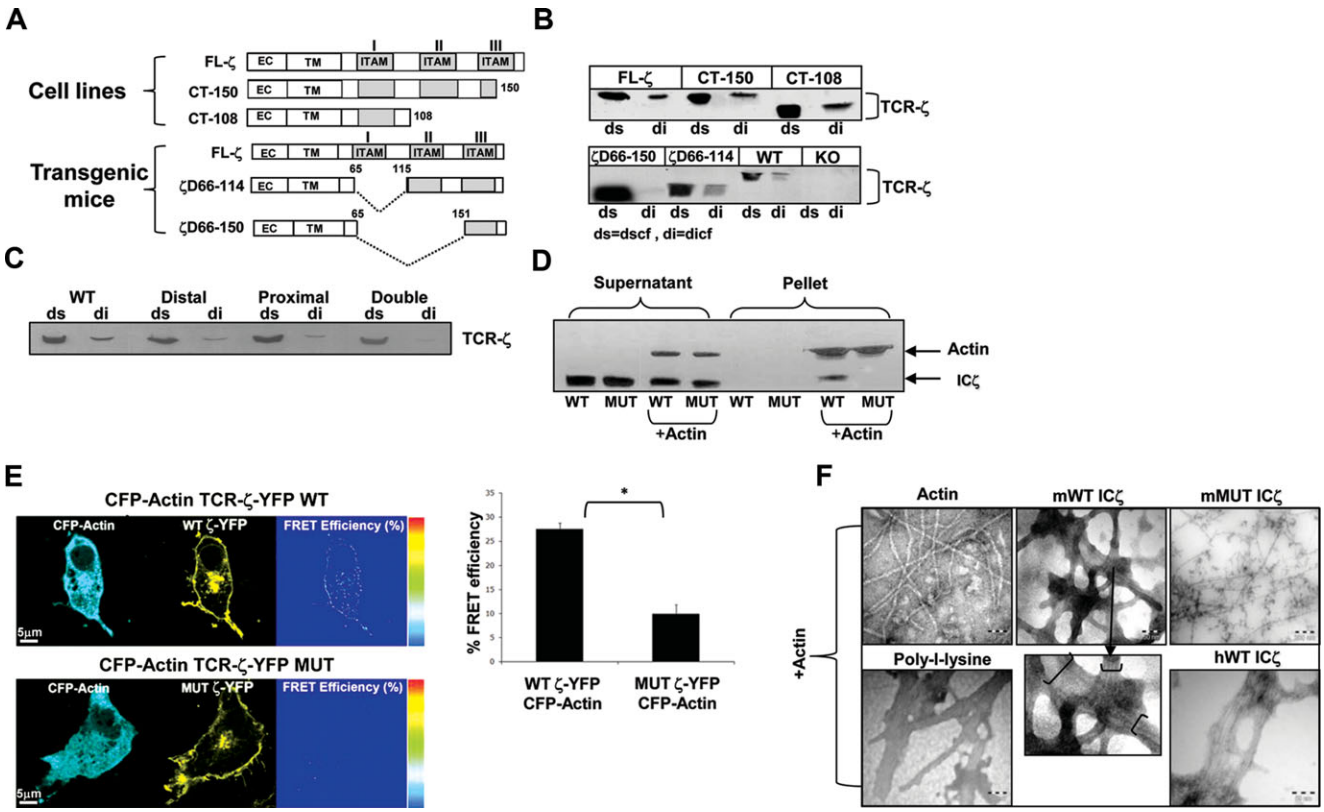

Figure 1. The two positively charged ζ motifs mediate its direct binding to actin and induce actin bundling. (A) Schematic representation of the full-length (FL) ζ structure and its truncated forms in cell lines (CT-150 and CT-180) and transgenic mice [deletion of aa 66–114 (ζ−D66−114) and aa 66–150 (ζ−D66−150)]. (B) Localization of ζ in dicf and dscf. T-cell lines expressing ζ, described in (A, top) and splenocytes from healthy C57BL/6 mice (WT), ζ KO, and transgenic mice described in (A, bottom), were lysed, dicf and dscf were separated and subjected to immunoblotting with anti-ζ Abs. A representative experiment out of three performed is shown. (C) COS cells transfected with WT ζ, or ζ mutated in its RRR motifs: Distal, Proximal, or double mutants, were lysed, dicf and dscf were separated and subjected to immunoblotting with anti-ζ Abs. A representative experiment out of three performed is shown. (D) Mutations of the positively charged motifs prevent cosedimentation of ζ with F-actin. F-actin was mixed with WT or MUT intracytoplamic (IC) regions of the ζ proteins, and analyzed for cosedimentation. (E) ζ associates with actin within cells. YFP-ζ (WT or MUT) and CFP-actin were co-expressed in COS cells and FRET analysis was performed as described in the section "Materials and Methods." Scale bar, 5 μm. Data are shown as mean + SD of 44 cells expressing WT ζ and 46 cells expressing mutated ζ imaged from three independent experiments (bottom). $^*p < 0.0001$, Student's *t* test. (F) ζ induces actin bundling via its positively charged motifs. Electron micrographs (magnifications of 220k) of negatively stained F-actin after incubation, alone, or with: poly-lysine (positive control), IC regions of WT human (hWT IC ζ), WT mouse (mWT IC ζ), or MUT mouse ζ (mMUT IC ζ). The massive bundling induced by mWT IC ζ is digitally enlarged and presented in the middle low panel (arrow), wide bundles are indicated. A representative experiment out of at least three performed is shown.

precipitated with F-actin (Fig. 1D). Testing the capacity of WT and MUT ζ IC domains or peptides represent the described WT and MUT motifs, to bind F-actin showed that only the WT IC ζ protein or the peptide containing both RRR motifs could bind F-actin (Supporting Information Fig. 3). These results indicate that ζ can directly and specifically interact with F-actin, and that the positively charged motifs are crucial for this linkage.

We next determined whether ζ can associate with actin within cells and assessed the involvement of its basic motifs. To this end, we used fluorescence resonance energy transfer (FRET) technology. First, to establish the use of sensitized emission FRET, we employed cells expressing yellow fluorescent protein (YFP) conjugated to cyan fluorescent protein (CFP) as positive control and cells expressing CFP and YFP separately. FRET was detected in the positive control cells (47.4% ± 1.6) but not in the negative control cells (0%; Supporting Information Fig. 4A). Subsequently, we tagged WT and MUT ζ with YFP and actin with CFP, and expressed them in COS7 cells at the same level (Supporting Information Fig. 4B). FRET analysis was performed in order to follow the interaction between actin and WT ζ in comparison with MUT ζ. Our data indicate that WT ζ associates with actin, as demonstrated by the high FRET efficiency (27.5% ± 1.3) for this interaction (Fig. 1E). However, FRET efficiency between actin and ζ was significantly reduced (9.9% ± 1.9) in cells expressing the MUT ζ, indicating the major role of ζ basic motifs in the molecular interaction with actin. To verify these results we performed an acceptor photobleaching FRET assay. Our results indicate that the trend observed in the donor-sensitized acceptor fluorescence emission FRET analysis was maintained since a significantly higher relative FRET efficiency was observed in cells expressing WT ζ WT versus MUT ζ MUT(Supporting Information Fig. 4C).

To assess whether ζ has a structural effect on actin reorganization, we hypothesized that the positively charged ζ motifs could be involved in actin bundling, as observed for various proteins containing positively charged clusters [15, 16]. To this end, F-actin was mixed with different concentrations of WT or MUT IC ζ proteins, stained and analyzed by electron microscopy. As shown in Fig. 1F, while actin filaments incubated alone appear individually dispersed and disorganized in the field, addition of the WT mouse (mWT ICζ) or human (hWT ICζ) proteins induced actin organization and formation of bundles that appear as wide branches (lower middle panel) similar to those induced by the positively charged poly-l-lysine. In contrast, when the MUT ICζ was added, a disorganized actin microfilament field is observed. These results indicate that the two ζ chain RRR motifs of the mouse and human origin mediate not only the direct association with actin but also induce bundling of actin filaments.

ζ Basic motifs mediate TCR–cytoskeleton association and are required for IS formation

We next analyzed whether the ζ basic motifs are also responsible for its association with the cytoskeleton within T cells. To this end, we stably expressed the full length (WT) or the double mutated (MUT) ζ in ζ−deficient hybridoma T cells, which lack TCR cell surface expression. Both WT and MUT ζ−expressing cells restored TCR surface expression (Supporting Information Fig. 5A), suggesting a normal association between the WT and MUT ζ and the remaining TCR subunits. Moreover, immunoprecipitation of ζ from WT and MUT cells using anti-ζ Abs ("a"–"d"), directed against different epitopes within the ζ IC region, depicted similar ζ levels precipitated from both cell types (Supporting Information Fig. 5B and C). These indicate that the ζ mutations did not affect its conformation. In all comparative experiments WT and MUT expressing cells expressed similar cell surface TCR levels. To assess the effect of ζ mutations on its association with the cytoskeleton, we compared the distribution of the cska- and non-cska-TCR forms between the two cell types. Total non-cska ζ levels in both WT- and MUT-expressing cells were similar to those of the parental ζ−expressing 2B4 cells from which the ζ-deficient T cells were derived (Fig. 2A). However, mutations in the basic motifs disrupted the ζ cytoskeleton association, resulting in a pronounced impairment of the cska-TCRs, with only a negligible expression (Fig. 2A). To verify the results obtained using hybridoma T cells, we transfected primary splenic and thymic T cells, isolated from ζ knockout (KO) mice [17], with WT and MUT ζ cDNAs. The results revealed a similar effect of the mutations on the ζ–cytoskeleton interaction (Fig. 2B), as observed when using the MUT cells (Fig. 2A). The CD3ε chain followed the distribution of ζ, as it was not found only in the cytoskeletal fraction of the MUT cells (Fig. 2C). These results suggest that the association between the TCR subunits and the cytoskeleton is mediated via ζ, and that the positively charged ζ motifs are responsible for this linkage. We further demonstrate that ζ is also associated with actin within cells; while WT ζ is co-immunopercipitated from cell lysates via anti-actin Abs, MUT ζ was undetected (Supporting Information Fig. 5D). Similar results were obtained when comparing the ζ–actin interaction between splenocytes from WT and ζ−D66−150 mice, the latter lacks the two positively charged motifs; only the WT ζ was found associated with actin (Supporting Information Fig. 5D). Together, these data indicate a ζ–actin association within T cells, which is mediated via the two positively charged motifs.

Next, the role of the cska-TCRs in T-cell activation was assessed. The above-described data showing that ζ induces actin bundling (Fig. 1F) suggest a structural role for the cska ζ in IS formation/maintenance. To test this possibility, we first analyzed the polar TCR clustering that follows TCR-mediated activation, which is an early step in IS formation. T cells stably expressing WT or MUT ζ were activated with anticlonotypic Ab-coated beads and subjected to immunostaining using anti-CD3ε Abs. Confocal microscopy analyses revealed that the MUT cells were unable to display polar TCR clustering upon TCR cross-linking, as opposed to the cells expressing the WT ζ (Fig. 2D). Despite the inability of the MUT cells to display TCR clustering, they could still transmit immediate TCR-mediated signaling events similar to the WT cells, as indicated by the induction of ζ isoforms (phosphorylated and ubquitinated) [18] and ZAP-70 and LAT phosphorylation kinetics (Supporting Information Fig. 6A–C). Using a more physiological activation condition in which peptide loaded APCs were incubated with WT and MUT T cells expressing the corresponding specific TCR, revealed similar results; the MUT cells could not display a polar TCR clustering and IS formation (Fig. 2E). These results indicate that cska ζ have a key role in the immediate creation and maintenance of TCR clustering that evolves to IS.

cska-TCRs play a fundamental role in optimal T-cell and APC activation

We next assessed the significance of the cska-TCRs in the outcome of TCR-mediated activation. It was previously demonstrated that TCRs undergo extensive lysosomal degradation following activation, leading to depletion of surface TCRs and intracellular reservoirs [19]. However, while most studies focused on the non-cska-TCRs, the cska-TCRs were largely neglected. Herein, we demonstrate that both cska-TCRs and non-cska-TCRs undergo a similar degradation process upon TCR-mediated activation (Supporting Information Fig. 7A). When T cells were activated in the presence of the lysosomal inhibitor NH_4Cl (Supporting Information Fig. 7B and C), expression of both TCR forms was rescued. Surprisingly, under these conditions cska-TCRs accumulated at much higher levels (up to 200% of their expression in the nonactivated state) than the non-cska-TCRs (up to 75% of their expression levels in nonactivated cells), in the same cells (Supporting Information Fig. 7B and C). Levels of the T-cell-specific ZAP-70 PTK, which served as control, were unchanged (Supporting Information Fig. 7B). These results suggest that following activation most TCRs become associated with the cytoskeleton.

Despite the massive downregulation of cell surface-expressed TCRs upon activation, low levels of surface receptors are

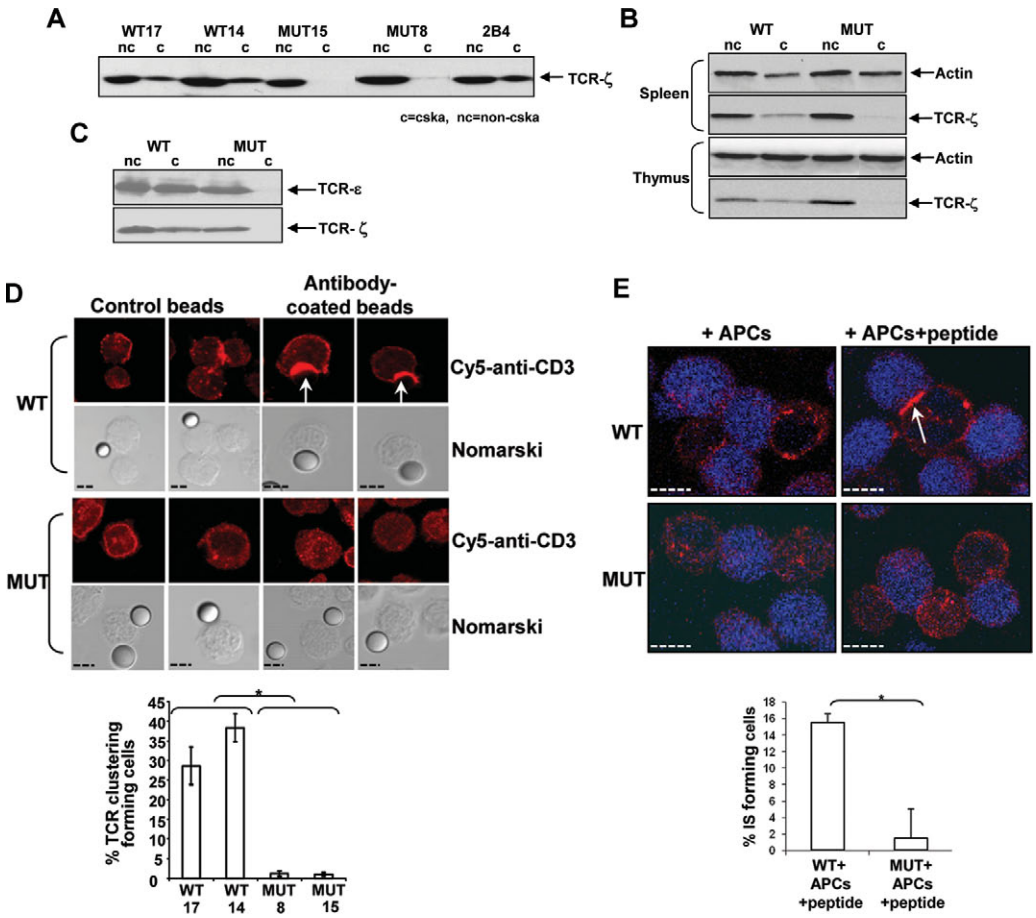

Figure 2. ζ positively charged motifs play a key role in its association with the cytoskeleton and in TCR clustering and IS formation. (A–C) T cells expressing MUT ζ are devoid of cska-TCRs. (A, C) ζ-deficient T-cell clones stably expressing the WT (17 and 14) or MUT (8 and 15) ζ or (B) purified T cells from the spleen or thymus of ζ KO mice transiently expressing the WT or MUT ζ were lysed, cska and non-cska fractions were separated, and immunoblotted with anti-ζ (A, B) or anti-ε Abs (C). A representative experiment out of at least three performed is shown. (D) ζ RRR motifs are required for TCR clustering formation induced upon T-cell activation. WT and MUT ζ expressing T-cell clones were activated with control- or Ab (A2B4)-coated beads and anti-CD28 Abs, fixed, stained with anti-CD3ε Abs, and analyzed by confocal microscopy. Scale bar, 6 μm. TCR clustering formation was scored as described in the section "Materials and Methods." Data are also shown as mean + SD of three independent experiments (bottom). $^*p < 0.0001$, Student's *t* test. (E) ζ RRR motifs are required for IS formation induced upon T-cell activation. WT and MUT ζ expressing T-cell clones were incubated with tracker labeled APCs (blue) in the absence or presence of cytochrome C peptide. The cells were stained for TCR expression and analyzed by confocal microscope as in (D). Scale bar, 6 μm. Data are also shown as mean + SD of three independent experiments (bottom); $^*p < 0.0001$, Student's *t* test.

maintained for the completion of T-cell activation [11]. However, the identity of these stably expressed surface TCRs remained unknown. We demonstrate that while levels of cell surface expressed non-cska-TCRs were dramatically reduced following activation, levels of cell surface expressed cska-TCRs were only slightly reduced (Fig. 3A, left panel). Thus, the majority of TCRs expressed on the surface of activated T cells (after 14 h) belong to the cska population, despite the total recovery of both TCR populations to normal levels within the cell (Fig. 3A, right panel).

We next followed the effect of cska-TCRs on the outcome of long-term activation and assessed their effect on the capacity of the WT and MUT cells to secrete cytokines (IL-2) upon TCR-mediated activation. The results revealed that the MUT cells secreted significantly less IL-2 than the WT cells (Fig. 3B). Upon activation with PMA and ionophore, which bypass the TCR, no differences between the MUT and WT cells in IL-2 secretion were observed, indicating a similar capacity of both cells to produce/secret IL-2 when activated via pathways that circumvent the TCR (Fig. 3B). Assessment of the capacity of the WT and MUT cells to synthesize cytokines revealed that the MUT cells synthesized significantly lower amounts of IL-2 compared with the WT cells (Fig. 3C). In addition, differences between MUT and WT cells were also observed in the induction of the cell surface expressed activation-dependent markers, CD25 and CD69 (Fig. 3D and F). Moreover, we also demonstrate that successfully activated WT T cells can affect the corresponding APCs, leading to the induction of CD25 and CD69 on their cell surface (Fig. 3E and G). In contrast, activated MUT T cells did not support CD25 and CD69 induction on the APCs (Fig. 3E and G), most likely due to the lack of IS formation and aberrant MUT cells activation.

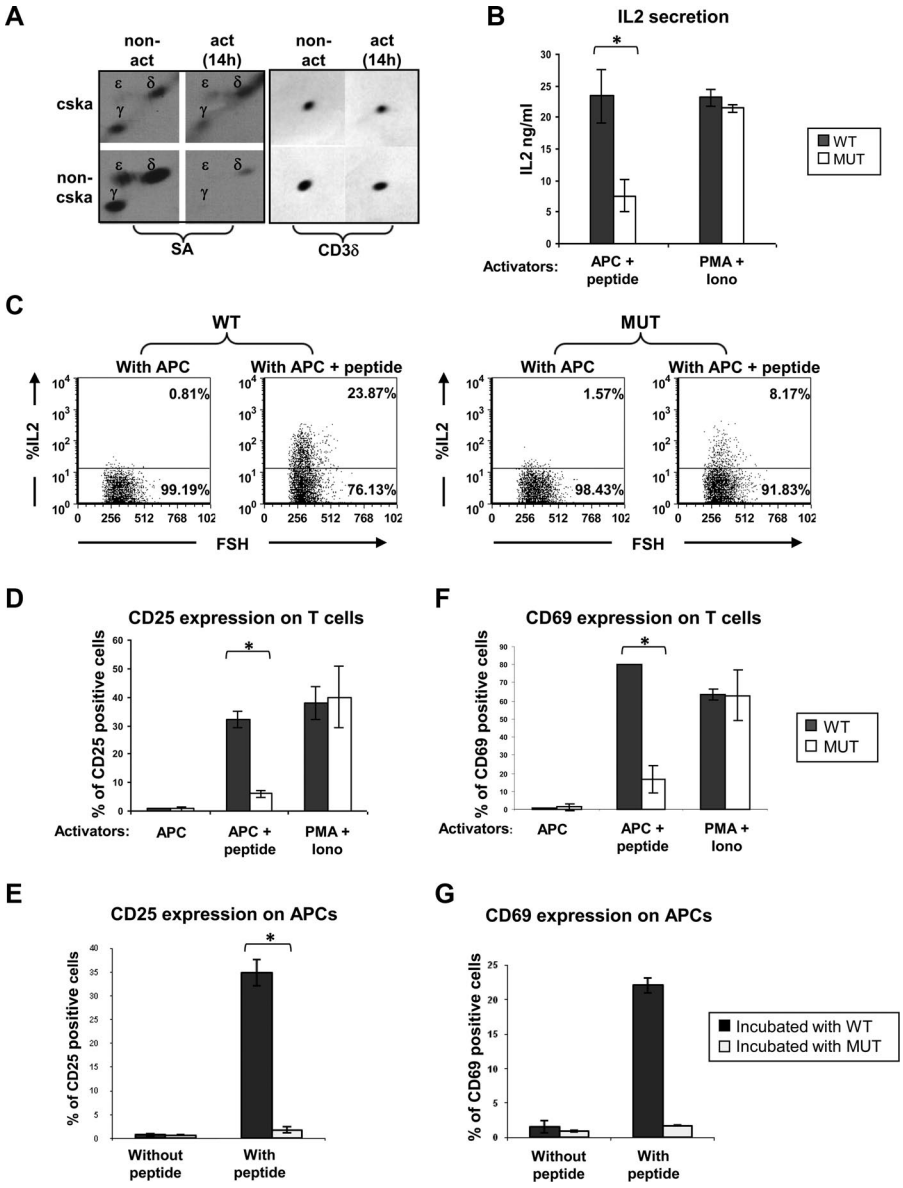

Figure 3. Mutations of the ζ actin-binding motifs lead to aberrant long-term T-cell and APC activation events. (A) Cska-TCRs are the receptors expressed on the cell surface following extended activation. Splenocytes were activated for 14 h, subjected to cell surface biotinylation, lysed, and cska and non-cska proteins were separated. TCRs were immunoprecipitated and resolved on 2D nonreducing/reducing SDS-PAGE. Cell TCRs were detected using strepavidin-HRP (SA). CD3δ subunit expression levels were measured by immunoblotting using anti-δ AbsAbs to estimate the total TCR amount. One representative experiment out of four performed is shown. (B) WT and MUT T cells were activated for 16 h with APCs loaded with cytochrome C peptide or with PMA+Ca ionophore. Supernatants were collected and analyzed by ELISA to determine secreted IL-2 levels; $^*p < 0.045$. (C) Cells activated as in (B), and were stained for IL-2 production and analyzed by FACS, gating on T cells (see gate strategy in Supporting Information Fig. 7). A representative experiment is present out of three independent experiments performed. (D–G) Cells were stained for surface (D, E) CD25, or (F, G) CD69, gating on T cells. (D) $^*p < 0.007$; (F) $^*p < 0.01$; or on the APCs (E) $^*p < 0.003$, (G) $^*p < 0.001$, Student's *t* test. All data are shown as mean ± SD of data pooled from three independent experiments.

Discussion

Dynamic regulation of TCR expression levels, TCR membrane reorganization, and interaction with intracellular molecules are key processes in modulating T-cell responses. Upon Ag recognition TCRs become clustered at the T-cell–APC contact area, joining a well-organized IS [3], and are subsequently internalized and degraded, resulting in a reduced TCR-cell surface expression [19]. Despite the low TCR-cell surface levels, TCR-mediated signaling continues for up to 10 h, and polarized cytokine secretion occurs even later [11]. These events are associated with a dramatic polymerization and polarization of actin microfilaments, which is critical for IS establishment, T-cell activation, and execution of effector functions [7, 20]. The maintenance of IS required for full T-cell activation and the observed polar dynamics of actin toward the IS, raise key questions about the molecular basis for the specificity and stability of such a prolonged interaction. We hypothesized that the dicf-TCRs, could potentially play a role in the specific prolonged maintenance of the IS generated in the course of T-cell activation.

Herein we are the first to show that of all TCR subunits, only ζ possesses two RRR clusters within its IC region, which mediate its direct binding to F-actin, enabling a steady expression of the dicf-TCRs, which we proved to be cska-TCRs. Positively charged residues, when appropriately exposed on the surface of a protein can bind to negatively charged actin filaments [15]. By using sedimentation assays and FRET analysis, we demonstrate that while WT ζ can directly bind F-actin, the MUT protein lacking the two motifs is unable to do so. Moreover, EM analyses revealed that both human and murine ζ have the capacity to induce F-actin bundling via the two positively charged clusters. However, ζ mutated in its two motifs was devoid of this ability. The in vivo

appearance of ζ as a homodimer could enhance its potency to bundle actin within cells. In most cellular structures constructed by actin bundles, more than one actin-bundling protein is present [21]. This rule is apparently maintained for T-cell IS formation, as shown for the actin-bundling proteins, α-actinin [22], and the Tec family PTK, Itk [23]. Thus, cska ζ in conjunction with numerous actin cross-linking proteins may cooperate in shaping the IS by serving as a core/anchor for actin bundling.

Our results indicate that ζ association with actin plays an essential role in TCR-mediated T-cell membrane structural changes and distal activation processes. T cells expressing ζ mutated in its two RRR motifs, although having similar levels of cell surface expressed TCRs as that of the WT, are devoid of cska-TCRs. In these MUT cells TCRs are unable to associate with actin or form activation-induced TCR clustering when compared with the WT cells. Upon activation, TCR microclusters associated with intracellular signaling molecules are induced toward the interacting APC. The presence of ζ in the TCR, its linkage to actin in resting T cells, and its ability to induce actin bundling, enable it to play a unique role in the induction of specific polar spatial organization of actin filaments into a network that interacts with the membrane. These changes lead to an IS arrangement and receptor-mediated signalosome formation [1–3]. Supporting evidence for the importance of ζ in IS formation reside also in a study showing that IC ζ region fused to the TCRαβ subunits is sufficient to enable the induction of IS in the absence of the invariant CD3 subunits [24]. Moreover, we demonstrate that steady levels of cska-TCRs are expressed on the cell surface throughout a long-term activation process, even though they are subjected to lysosomal degradation. This phenomenon is most likely due to the large pool of this receptor form accumulated within cells during activation. This is in contrast to the non-cska-TCRs that are degraded upon activation and are practically absent from the T-cell surface. These results suggest that sustained TCR-mediated signaling [11] observed even after the majority of receptors have been degraded is due to the cska-TCR population.

Our data and the cumulative knowledge on IS formation and maintenance at the T-cell–APC contact interface lead us to assess the effect of the mutated ζ on immediate and long-term activation processes. We found that although the MUT cells are capable of initiating immediate TCR-mediated signaling events as reflected by the induction of cska ζ isoforms, ZAP-70 and LAT phosphorylation, they synthesized and secreted significantly less IL-2 when compared to the WT cells. These results suggest that the proximal TCR signaling pathway is uncoupled from distal events following modulation of the actin cytoskeleton binding due to the ζ mutations.

Following TCR-mediated activation, the MUT cells as well as their corresponding APCs, expressed much lower levels of the CD25 and CD69 activation markers, when compared with the WT cells and their activating APCs. CD25 and CD69 are expressed on T cells and other leukocytes 3 to 16 h following activation [25]. Thus, lack of IS formation in the MUT cells disables "cross talk" between the cells, and results in a weak stimulation and aberrant long-term activation of both T cells and APCs. Interestingly, recent studies reported that ζ possesses various positively charged phosphoinositide-binding residues of which in part overlap with the RRR motifs described herein [26–28]. In these studies, mutations in such residues impaired TCR clustering, similarly to our results when mutating the two RRR motifs. Thus, binding phosphoinosidies and actin within the cell could be mediated in parallel by positively charged motifs positioned at various regions of ζ and affect IS formation. However, of particular significance are the two RRR motifs we have identified since we found that they mediate the association between the TCR and the cytoskeleton in resting and activated T cells and are required for IS maintanace for the execution of long activation events, while the mutations described by Zhang et al. [28] showed dissociation of ζ from the membrane upon activation and the role in IS formation and maintenance was not discussed. Thus, both observations strongly suggest that both interactions play a key role in T cells but are most likely differently coordinated and could appear jointly or separately under specific resting/activation T-cell conditions. Indeed, recent studies described the significance of such interactions [29]; that plasma membrane phosphoinositides play a central role in regulating the organization and dynamics of the actin cytoskeleton by acting as platforms for protein recruitment, triggering signaling cascades and directly regulating the activities of actin-binding proteins. One could speculate that the ζ chain could serve as an adapter molecule linking between the plasma membrane and the actin microfilaments. Assessing the potential synergy of both interactions is expected to open new and important directions toward the understanding of T-cell activation processes.

T cells devoid of cska-TCRs resemble normal T cells treated with agents that disrupt actin polymerization [7, 30], and cells that were mutated in signal transduction proteins as VAV and ITK, which are also involved in actin-based cytoskeleton rearrangement upon TCR-mediated activation [4, 31]. Interestingly, the features of T cells lacking cska-TCRs, due to the expression of ζ mutated in its two RRR motifs, were similar to those observed in cells isolated from a chronic inflammatory environment characterized by immunosuppression and a massive ζ downregulation, while the remaining TCR subunits are expressed normally [32]. Our preliminary results indicate that under such conditions the cska-TCRs are the primary receptors dramatically downregulated, resulting in impaired TCR-mediated TCR clustering and IS formation, leading to T-cell dysfunction (data not shown). These initial data support the in vivo significant role of the cska-TCRs in T-cell activation processes. Further studies are required to explore this phenomenon due to its critical implication in various chronic inflammatory pathologies as cancer, autoimmune, and infectious diseases, all characterized by partial or severe T-cell immunosuppression [33].

In conclusion, our novel results suggest a model (Fig. 4) for the unique role of the cska-TCRs in resting and activated T cells. The cska ζ via the two positively charged motifs enables maintenance of a physical link between plasma membrane TCRs and actin in resting T cells, which is absent in the MUT cells (Fig. 4A).

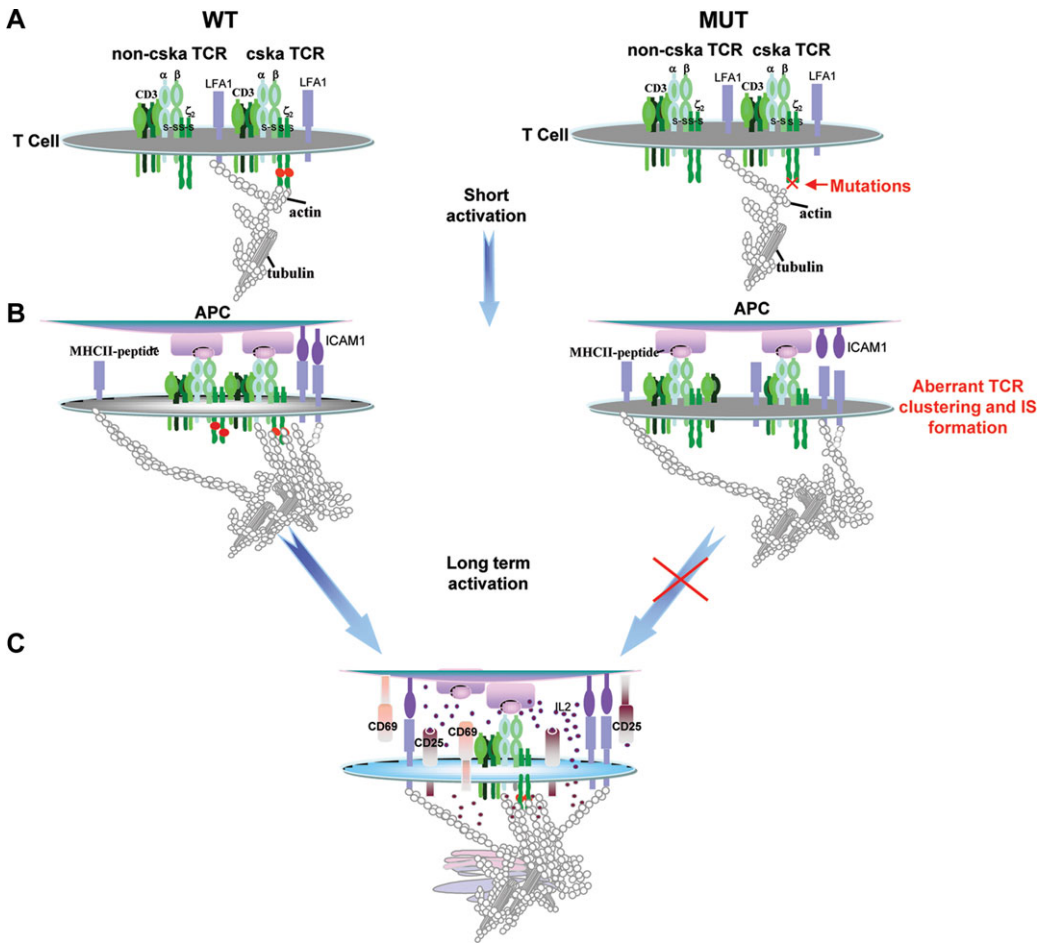

Figure 4. A proposed model for the roles of cska-TCRs in resting and activated T cells. (A) In resting WT T cells, cska-TCRs mediate an initial physical plasma membrane–cytoskeleton linkage via the two ζ positively charged motifs (red symbols). In the MUT cells, this linkage is absent. LFA1 is also linked to the cytoskeleton at the resting stage. (B) Upon TCR cross-linking, during the short-term activation period, IS is formed in WT T cells (white region of the cell membrane), the cska-TCRs directly lead actin bundling, possibly by transmitting immediate signals. Under such conditions LFA1 avidity to its ligand, ICAM1, increases. In contrast, these events are absent from the MUT T cells (C). During the late stages of activation (long-term activation), while the non-cska-TCRs are downregulated from the cell surface, cska-TCRs remain stably expressed, and in conjunction with LFA1 molecules [35], facilitate a specific long-lived polarization of the cytoskeleton and a sustained T-cell–APC interaction, resulting in the production and polar secretion of cytokines (small purple symbols) toward the T-cell–APC interface, and the activation of T cells and APCs, as indicated by expression of CD25 and CD69 on both cells.

This linkage, allows an immediate interaction of TCRs with the cytoskeleton upon Ag recognition. During immediate stages of activation (Fig. 4B), cska-TCRs in the WT cells play a dual role: (i) inducing physical changes that affect reorganization of both the cytoskeleton (actin bundling) and the plasma membrane profile (TCR clustering and IS formation), and (ii) initiating immediate signaling events, directly affecting the cytoskeleton. In contrast, these events are absent from the T cells expressing the MUT ζ. At a later stage of activation (Fig. 4C), while the non-cska-TCRs are downregulated from the cell surface, the cska-TCRs, by their stable expression at the cell surface, play an additional role in allowing transmission of continued T-cell activation signals. The cska-TCRs, in conjunction with surrounding adhesion molecules as LFA1 and CD2 [34, 35], and additional bundling proteins, maintain the specific polar orientation of cytoskeleton structures and a sustained T-cell–APC interaction. These are necessary for optimal cytokine synthesis and polar secretion toward the T-cell–APC interface, events critical for the activation of the T cells and the corresponding APCs, as indicated by expression of CD25 and CD69 on both cell types.

The presented model demonstrates the pivotal role of the cska-TCRs in resting T cells and in both early and late processes of T-cell activation. Moreover, our novel results fill the missing gap that was puzzled by numerous studies, aiming at understanding the mechanism underlying IS formation and maintenance, by showing that the TCR is directly connected to the cytoskeleton and that the cska ζ "guide" the initial activation signal via the TCR toward a subsequent actin-dependent receptor cluster formation.

Materials and methods

Mice

Female BALB/c mice were bred in the Hebrew University SPF facility. ζ KO and transgenic ζ DISTAL and TAIL-LESS mice were kind gift of Dr. W.E. Shores from the NIH [13].

Cells and Abs

Splenocytes were isolated from 6- to 12-week-old mice. 2B4 T-cell hybridoma and its ζ-deficient variant (MA5.8) expressing full length (FL) and truncated (CT-150 and CT-108) ζ were used. The Abs used are: A2B4 clonotypic Abs, anti-CD3ε, and anti-ζ, as previously described [8], anti-ZAP70 was a gift from L.E. Samelson (NIH), anti-CD3δ, anti-GST-LAT, and anti-GST were generated in rabbits, anti-Thy1.2 Abs (Serotek), anti-CD3ε, anti-CD28, anti-CD25, anti-CD69, and anti-IL-2 (BD Pharmingen), anti-CD16/32 and H57 (Biolegend), anti-phosphotyrosine (4G10) (UBI), anti-actin, and anti-pLAT (Abcam), Streptavidin-Cy5 or-allophycocyanin (Jackson Immunoresearch). Polyclonal Abs, "b", "c", and "d", directed against different epitopes within ζ, were generated in rabbits, and H-l46 anti-ζ (Ab "a") Abs were generously provided by Ralph Kubo, USA.

Cell fractionation, immunoprecipitation, surface biotinylation, splenocyte activation

dscf and dicf were separated from tested cells and when indicated, proteins were immunoprecipitated. Samples were separated on 1D or 2D nonreducing/reducing SDS-PAGE and subjected to Western blot analysis. The above-mentioned procedures and those for biotinylation and activation of splenocytes were previously described [10]. Ezrin and IκB were used in all experiments as control proteins to verify efficacy of detergent-insoluble and -soluble fractionation, respectively, and the ratio between dscf and dicf proteins were determined by densitometry analysis.

DNA constructs and transfection

Site-directed mutagenesis of murine ζ was performed using Pfu DNA polymerase (Stratagene) according to the manufacture's protocol. Double mutated (MUT) cDNA was sequenced and cloned into pcDNA3 (Invitrogen) for transfection or into pGEX6p2 to generate GST recombinant proteins. Transfection of WT and MUT ζ cDNA into ζ-deficient cells was performed by electroporation using Bio-Rad Gene Pulser II or Amaxa™ (Lonze Group Ltd. Switzerland). For FRET analysis, the WT and MUT ζ cDNAs were cloned into the Clontech expression vectors pEYFP-N1 to obtain YFP-tagged ζ proteins, and actin to pECFP-C1 to obtain the CFP-tagged actin. The actin plasmid was cotransfected into COS-7 cells (Lipofectamin 2000) with either WT or MUT ζ.

Cosedimentation assay and electron microscopy

G-actin was prepared from rabbit muscles and polymerized when required as previously described [36]. For cosedimentation, tested protein was added to prepolymerized F-actin, incubated for 20 min at 25°C and centrifuged at 80 000 rpm for 1 h at 4°C. Supernatants and pellets were separated, resolved on SDS-PAGE, and stained with Coomassie brilliant blue. For EM, samples were fixed on carbon-coated grids and negatively stained with 1% uranyl acetate. The grids were viewed under a Jeol 100cx (Jeol-LTD. Tokyo Japan) scanning EM.

Cell activation, confocal microscopy, and FRET analysis

For cell activation, 5×10^5 cells coated with anti-CD28 Abs were mixed with an equal number of 6-micron diameter polystyrene beads (Polysciences Inc, PA, USA) precoated with A2B4 Abs. After brief centrifugation, samples were incubated for various time periods at 37°C, transferred to poly-l-lysine coated slides (Lab-Tek), fixed, washed, and stained for CD3 expression. Confocal analysis was performed using LSM 410 microscope (Carl Zeiss MicroImaging, Inc.). TCR clustering formation was scored as positive if at least one distinct cap was observed at the cell–bead contact area. At least 100 cells in contact with beads were counted, and the percent cap formation was calculated.

For specific T-cell activation, APCs (LK B-cells) were labeled with blue cell tracker CMAC (Molecular Probes), washed, and incubated with or without the specific peptide (cytochrome C, 81–104 aa). After washing, a 1:1 ratio of LK cells and WT or MUT T cells were mixed and incubated at 37°C for different time periods. Cells were seeded onto a chamber slides, fixed, washed, stained, and analyzed as described. In ex vivo experiments, splenocytes were activated with anti-CD3ε Abs and processed as described. TCR clustering was detected by using anti-TCRβ Abs (Biolegend).

FRET was measured by donor-sensitized acceptor fluorescence [37]. CFP (excitation, 458 nm; emission, 465−510 nm) was used as a donor and YFP (excitation, 514 nm; emission, 530 nm) as an acceptor. The results were verified by using the acceptor photobleaching techniques as previously described [38]. Detailed description is provided in the Supporting Information. FRET was corrected and the FRET efficiency was determined.

Activation markers and cytokine analysis

Both WT and MUT cells were activated for 16 h at 37°C with PMA (40 ng/mL) and Ca ionophore (1.5 μm; Sigma) or with LK cells loaded with Pigeon cytochrome C peptide. Following activation,

cells were washed, and assessed for CD25 and CD69 expression by FACS analysis. Intracellular staining for IL-2 was performed using BD Fixation/Permeabilization Kit, and samples were analyzed by FACS (see gateing strategy in the Supporting Information section, Fig. S8), using Cell Quest software (BD PharMingen). For cytokine secretion analysis, cells were activated as described and the supernatants were assayed for IL-2 using ELISA (PeproTech, Rocky Hill, NJ, USA).

Statistics

All data were presented as average ± standard deviation (SD). Statistical significance was determined by Student's t test; $p < 0.05$ was considered statistically significant.

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Abbreviations: **CFP**: cyan fluorescent protein · **cska-TCR**: cytoskeleton-associated TCR · **dicf**: detergent-insoluble cell fraction · **dscf**: detergent-soluble cell fraction · **FRET**: fluorescence resonance energy transfer · **IC**: intracytoplasmic · **IS**: immune synapse · **MUT**: mutated molecule · **YFP**: yellow fluorescent protein

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