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Review

The calcium feedback loop and T cell activation: How cytoskeleton networks control intracellular calcium flux ☆

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ABSTRACT

During T cell activation, the engagement of a T cell with an antigen-presenting cell (APC) results in rapid cytoskeletal rearrangements and a dramatic increase of intracellular calcium (Ca^{2+}) concentration, downstream to T cell antigen receptor (TCR) ligation. These events facilitate the organization of an immunological synapse (IS), which supports the redistribution of receptors, signaling molecules and organelles towards the T cell–APC interface to induce downstream signaling events, ultimately supporting T cell effector functions. Thus, Ca^{2+} signaling and cytoskeleton rearrangements are essential for T cell activation and T cell-dependent immune response. Rapid release of Ca^{2+} from intracellular stores, e.g. the endoplasmic reticulum (ER), triggers the opening of Ca^{2+} release-activated Ca^{2+} (CRAC) channels, residing in the plasma membrane. These channels facilitate a sustained influx of extracellular Ca^{2+} across the plasma membrane in a process termed store-operated Ca^{2+} entry (SOCE). Because CRAC channels are themselves inhibited by Ca^{2+} ions, additional factors are suggested to enable the sustained Ca^{2+} influx required for T cell function. Among these factors, we focus here on the contribution of the actin and microtubule cytoskeleton. The TCR-mediated increase in intracellular Ca^{2+} evokes a rapid cytoskeleton-dependent polarization, which involves actin cytoskeleton rearrangements and microtubule-organizing center (MTOC) reorientation. Here, we review the molecular mechanisms of Ca^{2+} flux and cytoskeletal rearrangements, and further describe the way by which the cytoskeletal networks feedback to Ca^{2+} signaling by controlling the spatial and temporal distribution of Ca^{2+} sources and sinks, modulating TCR-dependent Ca^{2+} signals, which are required for an appropriate T cell response. This article is part of a Special Issue entitled: Reciprocal influences between cell cytoskeleton and membrane channels, receptors and transporters. Guest Editor: Jean Claude Hervé.

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1. Introduction

Host protection both from pathogens, such as viruses and bacteria, and from cancer is mediated by the immune system. As the first line of defense, cells of the innate arm of the immune system e.g. macrophages and dendritic cells, recognize and respond to pathogens in a non-specific manner. Cells at the site of infection then evoke an inflammatory response by releasing cytokines. Among other functions, these cytokines promote the recruitment and activation of both additional innate immune cells and cells of the acquired immune system, e.g. lymphocytes. A key step in the activation of the acquired immune response is the priming of naïve T lymphocytes by specialized antigen-presenting cells (APCs). The engagement between a peptide-specific T cell antigen receptor (TCR) and an APC bearing its cognate peptide subsequently results in cell cycle progression and proliferation. Antigen-primed T cells then survey the periphery for infected or transformed cells carrying their cognate antigen. Once a T cell specifically recognizes its target cell, it polarizes towards the T cell–target cell interface, the immunological synapse (IS), secreting cytolytic granules and/or cytokines that mediate the elimination of the malignant or infected target cell.

Hallmarks of T cell conjugation with an APC/target cell are rapid cytoskeletal rearrangements and a dramatic increase of intracellular calcium concentration. These events support the polarization of the T cell towards its target, forming an IS, which enables the redistribution of receptors, signaling molecules and organelles towards the T cell–APC contact surface and induces downstream signaling events, ultimately supporting T cell effecter functions.

TCR engagement with peptides conjugated to major histocompatibility complexes (pMHCs) presented on APCs, leads to activation of signal transduction pathways that promote a rapid release of Ca^{2+} from the endoplasmic reticulum (ER) Ca^{2+} stores [1–3]. Ca^{2+} depletion induces the opening of Ca^{2+} channels residing in the plasma membrane, known as Ca^{2+} release-activated Ca^{2+} (CRAC) channels [4,5]. These channels enable a sustained influx of extracellular Ca^{2+} across the plasma membrane in a process termed store-operated Ca^{2+} entry (SOCE) [6].

Prolonged elevation of intracellular Ca^{2+} through CRAC channels is required for varied T cell functions, including proliferation, differentiation, maturation, gene transcription and cytokine production [7–9]. Interestingly, CRAC channels have been shown to be inhibited by Ca^{2+} ions [4,5,10–13]. Thus, CRAC channels themselves cannot enable the sustained Ca^{2+} influx required for T cell function.

Increased intracellular Ca^{2+} levels are necessary for rapid cytoskeleton dependent polarization, which involves F-actin rearrangement and microtubule-organizing center (MTOC) reorientation [14,15]. Since Ca^{2+} levels rise within seconds following TCR engagement, whereas actin rearrangements occur further downstream in the cascade, an intriguing question is whether cytoskeleton rearrangements induce feedback regulation of Ca^{2+} signaling. Here, we will address this issue.

On the other hand, there are actin rearrangements that are partially triggered by the formation of Ca^{2+}-independent complexes that influence ongoing Ca^{2+} flux [16–22]. Additionally, some evidence suggests that actin rearrangements may be part of the TCR triggering process itself and, therefore, precede Ca^{2+} flux [20,23–25]. In agreement with these observations, the inhibition of actin polymerization by cytochalasin D has been shown to reduce T cell Ca^{2+} mobilization and T cell activation, as indicated by IFNγ production [26]. These effects support the notion that the remodeling of the actin cytoskeleton is essential for Ca^{2+} signaling. However, the mechanisms underlying the linkage between cytoskeleton rearrangements and Ca^{2+} signaling are not entirely understood. Recent observations have provided additional insights into how cytoskeleton rearrangements control crucial activities, such as Ca^{2+} signaling.

In this review, we briefly summarize the known principles regarding actin polymerization in cells, and focus on the less well understood role of cytoskeleton remodeling in maintaining Ca^{2+} signaling required for full T cell activation.

2. TCR triggering leads to Ca^{2+} influx

The intracellular Ca^{2+} concentration in resting T cells is maintained at ~50–100 nM, whereas the extracellular Ca^{2+} concentration is ~1 mM, resulting in a ~10^4-fold resting concentration gradient of Ca^{2+} across the plasma membrane. Following the engagement of the TCR with a pMHC on an APC, the intracellular Ca^{2+} concentration can increase to ~1 μM [1] through the sequential operation of two interdependent processes: (i) Release of phospholipase C gamma1 (PLCγ1)-dependent intracellular Ca^{2+} stores, and (ii) extracellular Ca^{2+} influx through store-operated plasma membrane Ca^{2+} channels [1–3] (Fig. 1).

2.1. PLCγ1 signals to induce intracellular Ca^{2+} store release

TCR–pMHC engagement leads to the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytosolic side of the TCR–CD3 complex by the Src-family kinases, Lck and Fyn [27,28]. These phosphorylation events enable the recruitment and activation of the tyrosine kinase ζ-chain associated protein of 70 kDa (ZAP-70), which in turn, enhances the phosphorylation of the ζ-chain, promoting the formation of small protein aggregates, known as microclusters (MCs) [23,29–37]. These MCs, which function as integrated signaling machines, consist of multiprotein complexes that are essential for intracellular signaling pathways downstream of the TCR engagement, such as Ca^{2+}-mediated signaling [38–41].

Of particular importance in Ca^{2+} signaling is the recruitment and activation of the intracellular enzyme PLCγ. Two forms of this protein have been identified, PLCγ1 and PLCγ2, of which T cells express predominantly the PLCγ1 form [42]. Two tyrosines, 775 and 783, located between the carboxyl terminus Src homology (SH) 2 domain and the SH3 domain, are crucial for the enzymatic activation of PLCγ1 in vivo [43–46]. Following tyrosine 783 phosphorylation by interleukin-2 (IL-2)-inducible T-cell kinase (Itk), PLCγ1 undergoes a conformational change that involves an intramolecular interaction between the carboxyl terminus SH2 domain and the phosphorylated tyrosine 783 [47]. Furthermore, all three SH domains of PLCγ1 are essential for its efficient recruitment, phosphorylation and activation in T cells [48].

The recruitment and activation of PLCγ1 at T cell MCs depend on several signaling molecules, including linker for activation of T cells (LAT); SH2 domain-containing leukocyte protein of 76 kDa (SLP-76); Vav1, a guanine nucleotide exchange factor (GEF); Itk; and c-Cbl [36,49,50] (Fig. 1). Reduced PLCγ1 phosphorylation and impaired Ca^{2+} mobilization have been described in T cell deficient or impaired in these molecules [16,39,40,48,51–55]. Indeed, both T cell development and signaling are abolished in the absence of either LAT or SLP-76, demonstrating their essential role in signal propagation [56–60].

LAT is a transmembrane adaptor protein that phosphorylation of its tyrosines provides docking sites for the recruitment of SH2 domain containing proteins, including PLCγ1, Grb2, and Grb2-related adaptor protein (GADS). Furthermore, these interactions constitute a platform for an indirect association between LAT and SH3 domain ligands of these proteins, such as SLP-76, which binds GADS SH3 domain through its proline rich domain (PRD), and c-Cbl, which interacts with the SH3 domain of Grb2 [57,61–63].

A study investigating the phosphorylation sequence and kinetics of the individual tyrosines on LAT revealed that the kinetics of LAT tyrosine 132 phosphorylation are much slower than that of tyrosine 191. This delayed phosphorylation of LAT tyrosine 132 is thought to ensure the tight control of PLCγ1 activity, and thus, is important for the regulation of signaling pathways downstream of this protein, including Ca^{2+} signaling [56].

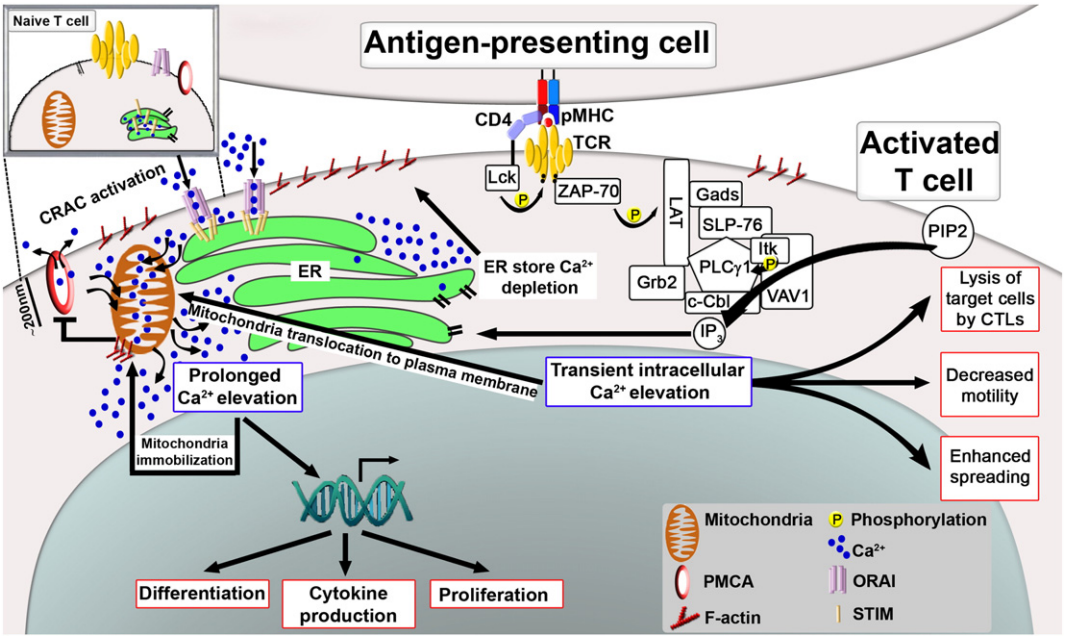

Fig. 1. Signaling cascades leading to cytoskeleton dependent nano-scale redistribution of specific organelles essential for T cell activation. Engagement of the TCR with a pMHC on an APC leads to the recruitment and activation of PLCγ1, which in turn, facilitates intracellular Ca^{2+} ER store release. The transient increase in cytosolic Ca^{2+} induces acute responses such as decreased motility, enhanced spreading, and lysis of infected target cells by cytolytic T cells (CTLs). This Ca^{2+} increase also promotes mitochondrial motility, and together with cytoskeletal dependent mechanisms, mitochondria are actively translocated towards the IS and are localized approximately 200 nm away from CRAC channels. Such close proximity between mitochondria and CRAC channels allows mitochondria to efficiently reduce the generation of local Ca^{2+} microdomains close to CRAC channels. Mitochondria at the IS also co-localize with the PMCA. This co-localization allows mitochondria to inhibit Ca^{2+}-dependent PMCA upregulation, resulting in decreased Ca^{2+} clearance by PMCA. Overall, this nano-scale redistribution of CRAC channels, mitochondria and PMCA serves to maintain the long-lasting global Ca^{2+} elevation required for generalized activation of T lymphocytes.

Similar to LAT, SLP-76, a cytosolic adaptor protein, undergoes phosphorylation, which promotes subsequent recruitment of SH2 domain-containing signaling molecules and is required for TCR-induced phosphorylation of PLC-γ1 [46,64]. These molecules include, among others, Vav1, and Itk [46,65–69]. Interestingly, it was found that elimination of Vav1 partially destabilizes the association between PLCγ1 and LAT, and elimination of SLP-76 abrogates the recruitment of PLCγ1 to LAT signaling complexes [48,70,71].

Thus, the recruitment of SLP-76 to the LAT-nucleated signaling complexes, through GADS, introduces a number of additional associated molecules into these complexes that are important for stabilizing PLCγ1-LAT interaction, and also appears to be essential for localizing Itk to the LAT complexes.

Itk, a member of the Tec family of tyrosine kinases, phosphorylates both PLCγ1 and SLP-76 on tyrosine 783 and 173, respectively, thus promoting the enzymatic activity of PLCγ1 [46,56,72–74]. Interestingly, ZAP-70, LAT, SLP-76, and Vav1 have also been found to be required for this phosphorylation [40,41,46,56,65,70,75]. The mechanism underlying Itk activation appears to involve its interaction with SLP-76. This interaction not only initiates Itk activity, but is also important for maintaining its kinase activity [46]. Itk has been shown to stabilize the Vav-SLP-76 interaction independently of its kinase activity [76], suggesting that Itk can function both as a tyrosine kinase and as an adaptor protein.

Following its phosphorylation, PLCγ1 hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to produce the second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [42,43]. DAG activates PKCθ and the MAPK/ERK pathways that induce the activation of the transcription factors activator protein-1 (AP-1), a transcriptional complex formed by c-Jun and c-Fos, and nuclear factor kappa B (NFκB) [77,78]. IP_3 binds to the IP_3 receptors on the ER membrane, triggering the release of Ca^{2+} stores from the ER. This release serves as the primary trigger for the opening of Ca^{2+} channels residing in the plasma membrane, enabling the sustained influx of extracellular Ca^{2+} across the plasma membrane (Fig. 1).

A recent study investigating actomyosin retrograde flow has revealed that perturbation of F-actin dynamics inhibits PLCγ1 phosphorylation within T cell MCs. This highlights the importance of ongoing actin polymerization, rather than a static F-actin scaffold, in promoting PLCγ1 activation and continuous Ca^{2+} signaling [79].

3. Different Ca^{2+} signaling patterns enable discrete T-cell effector functions

In immune cells, an increase in the intracellular Ca^{2+} concentration affects the regulation of various physiological events [7–9,80–85]. Different T cell effector functions are regulated by different durations and amplitudes of Ca^{2+} elevation. While short term responses such as decreased motility, enhanced spreading, and lysis of infected target cells by cytolytic T cells (CTLs) are induced by a moderate and transient increase in cytosolic Ca^{2+}, other diverse cellular functions including proliferation, cytokine production, differentiation of naive T cells into Th1, Th2 and Th17 effector subtypes, maturation, and gene transcription require intense and prolonged elevation of intracellular Ca^{2+}. Thus, Ca^{2+} is a versatile messenger that allows for different outcomes within the cell, depending on the magnitude and duration of changes in its concentration.

Recently, a novel regulatory mechanism of Ca^{2+} that amplifies TCR-induced signaling, in which Ca^{2+} binds directly to anionic phospholipids within the plasma membrane and neutralizes them, has been reported. Thus, Ca^{2+} interferes with the ionic interactions between positively charged CD3ε/ζ cytoplasmic domains and negatively charged phospholipids in the plasma membrane, allowing the dissociation of CD3ε/ζ from the membrane and the exposure of ITAMs, thus facilitating their phosphorylation. Rather than initiating CD3 phosphorylation, this regulatory mechanism of Ca^{2+} has a positive

feedback effect on amplifying and sustaining CD3 phosphorylation, and is also proposed to enhance T-cell sensitivity to foreign antigens [86].

The activation of different transcriptional pathways is dependent upon distinct Ca^{2+} signaling patterns. For example, the activation of the nuclear factor of activated T cells (NFAT) transcription pathway, which controls the transcription of critical effector molecules such as IL-2, requires low but prolonged intracellular Ca^{2+} elevation to induce the calcineurin dependent NFAT dephosphorylation needed for NFAT translocation to the nucleus [7,81,87,88]. A decrease in intracellular Ca^{2+} levels results in the immediate export of NFAT from the nucleus [87,89]. Therefore, transient Ca^{2+} entry is not sufficient to activate this transcriptional pathway. In contrast, NFkB, another transcription factor that regulates IL-2 expression, is translocated to the nucleus following degradation of its inhibitory subunit, IκB, and can be activated by stronger and shorter increases in Ca^{2+} concentration [78]. The reason for this selectivity derives from the differences in Ca^{2+} sensitivity of NFAT and NFkB, as well as from their activation and deactivation kinetics. NFAT activity is highly sensitive to Ca^{2+} levels, but is rapidly reversible, whereas NFkB activity has a relatively low Ca^{2+} sensitivity but long persistence after Ca^{2+} removal, due to its slow deactivation, resulting from the slow process of IkB synthesis [1,90].

Multiple feedback pathways involved in regulation of Ca^{2+} signaling lead to the widespread occurrence of Ca^{2+} oscillations in T cells, as well as in many other cell types [91]. Ca^{2+} oscillations in lymphocytes differ from those observed in most other cell types, in that they reflect fluctuations of Ca^{2+} entry rather than of Ca^{2+} release. The complete mechanisms underlying these oscillations are not fully understood. According to Dolmetsch et al., these oscillations involve delays between store emptying and CRAC channel activation as well as delays between store refilling and CRAC channel deactivation [92].

These oscillations have been suggested to maximize the sensitivity of T cells to weak stimuli [93,94]. Also, oscillations help to activate specific Ca^{2+}-dependent genes, as the activation of different transcription factors is affected by the frequency of oscillation. For example, while NFAT accumulation in the nucleus requires rapid oscillations, NFkB can accumulate in the nucleus even in response to infrequent oscillations [93,95,96]. This may account for differences between these transcription factors in their deactivation kinetics. Intriguingly, it is not clear whether transcriptional activity is determined by these oscillations or by the period of time in which Ca^{2+} is above a certain threshold [97].

Although the physiological importance of these oscillations is well understood, the linkage between Ca^{2+} oscillations to cytoskeletal machinery in T cells is unknown and requires investigation.

Taken together, these data suggest that the versatility of Ca^{2+} may serve to enhance the efficiency and the specificity of Ca^{2+} dependent downstream pathways, and is likely to add another level of regulation to the dynamics of Ca^{2+} signaling in T cells.

4. Ca^{2+} signaling is controlled by the dynamic operation of Ca^{2+} sources and sinks

Cellular Ca^{2+} signals result from the interaction of multiple Ca^{2+} sources, e.g. store-operated Ca^{2+} channels, and sinks, e.g. Ca^{2+} ATPase pumps and Ca^{2+}-consuming organelles [1].

Several ion channels that mediate Ca^{2+} influx in lymphocytes have been identified, including store-operated CRAC channels as well as P2X purinoreceptor channels, transient receptor potential (TRP) channels, and voltage-gated Ca^{2+} (CaV) channels [98], of which the CRAC channels appear to be the predominant antigen receptor-activated Ca^{2+} channels and the major Ca^{2+} entry pathway in T cells, as well as the most highly selective Ca^{2+} channels known [1,97,99,100]. Ca^{2+} influx through these channels elevates the intracellular Ca^{2+} concentration for minutes through hours. This phenomenon is termed SOCE, formerly also called capacitative Ca^{2+} entry.

CRAC channel blockers inhibit T cell activation and proliferation [97]. Consistent with this, Jurkat T cells with defective CRAC channels show corresponding impairment in calcium-dependent gene transcription. In addition, mutations that abolish CRAC channel expression or activity are linked with various immune-disorders, e.g. severe combined immunodeficiency (SCID) syndrome and increased susceptibility to severe infections [101–107]; these phenotypes emphasize the crucial importance of Ca^{2+} influx through CRAC channels for T-cell-dependent immune response.

Despite the indisputable importance of Ca^{2+} sources in T cell signaling, Ca^{2+} clearance from the cytosol by Ca^{2+} sinks is also of great significance, as it influences the amplitude, duration, and dynamics of Ca^{2+} signaling. Ca^{2+} pumps that mediate the removal of cytosolic Ca^{2+} in lymphocytes include sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), which pumps Ca^{2+} from the cytosol into the ER, and plasma membrane Ca^{2+} ATPase (PMCA), which pumps Ca^{2+} from the cytosol out of the cell [108–110]. The PMCA is a transmembrane transport protein [111]. This pump binds Ca^{2+} with high affinity, but has a low capacity for Ca^{2+}. Thus, PMCA finely regulates the concentration of cytosolic free Ca^{2+} around its basal levels. PMCA activation depends on Ca^{2+}-binding protein calmodulin. Binding of Ca^{2+}-loaded calmodulin to the carboxyl terminus of the PMCA activates the pump [112,113]. Calmodulin binding also enhances PMCA activity, increasing the dynamic range of its pumping activity, and efficiently supports Ca^{2+} homeostasis in the cell [114].

The ER has been extensively studied for years as the major store of Ca^{2+}. However, it is now clear that the ER is not the only controllable Ca^{2+} store within the cell. Other organelles also have the ability to provide Ca^{2+} storage, including the mitochondria, nucleus, Golgi and lysosome-related vesicles [115].

In the next sections, we focus on the CRAC channels as the major source of Ca^{2+} influx in T cells, and on mitochondria as a Ca^{2+} sink that can modulate the activity of the CRAC channels.

4.1. Store-operated CRAC channels induce most of the Ca^{2+} elevation in T cells

4.1.1. Molecular components of CRAC channels: STIM and ORAI

Although CRAC channels have been extensively characterized in terms of their electrophysiological properties [1,5,100,116], it was only in the last years that there has been a breakthrough in identifying their molecular components, and in the understanding of their activation mechanism.

CRAC channels consist of two major proteins, termed ORAI, also referred to as CRAC-membrane (CRACM), and the stromal interaction molecule (STIM), both of which are essential elements in CRAC channel activation [101,117–120]. Three isoforms of ORAI, designated ORAI1, ORAI2, and ORAI3 (CRACM1, CRACM2 and CRACM3, respectively), and two isoforms of STIM (designated STIM1 and STIM2) are found in mammalian cells [101,118,121–123]. Among these isoforms, ORAI1, as well as both STIM isoforms [124], appear to be the main channel proteins mediating Ca^{2+} influx in T cells [3,123]. However, the exact function of these various isoforms has yet to be fully established.

ORAI was discovered using the combination of two genetic approaches, a genome-wide RNA interference (RNAi) screen in *Drosophila* [101,119,120], and a linkage analysis in cells from patients with SCID lacking CRAC channel activity [101]. Very recently, the crystal structure of Orai from *Drosophila melanogaster*, which shares 73% sequence identity with human ORAI1 within its trans-membrane region, has been resolved. This crystal structure provides a better comprehension of the electrophysiological properties of CRAC channels, including their low

conductance, exquisite Ca^{2+} selectivity, ion permeation, as well as their gating mechanism [125].

ORAI1 is a 32.7 kDa, widely expressed glycoprotein, anchored in the plasma membrane through its four transmembrane α helices, where it forms the pore subunit of the CRAC channel [119,120,125–128]. Several lines of evidence suggest that the channel is formed by the assembly of several ORAI subunits [123,129–132]. Indeed, the recently resolved crystal structure of Orai, revealed that the channel is composed of a hexamer containing six Orai subunits arranged around a central axis [125].

Both the amino and carboxyl termini of ORAI1 are located in the cytosol. The carboxyl terminus contains negatively charged residues, within a coiled-coil domain, which constitute a binding site for STIM [133]. Deletion or mutation of this region abrogates both the interaction between STIM1 and ORAI1 and calcium influx [134–137].

Interestingly, a missense mutation in ORAI1, which involves a single amino acid substitution of arginine by tryptophan at position 91 (R91W), renders the channel non-conductive, and results in SCID, demonstrating the importance of CRAC channels, and ORAI1 in particular, in the in vivo immune response [101].

STIM was discovered using limited RNAi screens in both *Drosophila* and HeLa cells [117,118]. STIM is a 77 kDa single-pass transmembrane protein, located predominantly in the ER, such that its amino terminus resides in the ER lumen and its carboxyl terminus resides in the cytosol [117,138–140]. The amino terminus contains two EF hand domains, of which only one enables STIM to bind Ca^{2+}, and thus to sense the ER Ca^{2+} concentration, and a sterile α motif (SAM) domain [117,141–145].

4.1.2. CRAC channel activation

The generation of CRAC-mediated currents in cells requires a series of coordinated sequential steps, most of which are common to both immune and non-immune cells.

In resting cells, the ER Ca^{2+} stores are replete, and Ca^{2+} within these stores is bound to the EF hand domain of STIM1 proteins, which are diffusely distributed throughout the ER membrane [117,145] (Fig. 1). Ca^{2+} binding to the EF hand domain induces a closed inactive conformation, in which this domain and the second EF hand domain are folded through the formation of hydrophobic contacts with the SAM domain. Stimulation that promotes Ca^{2+} release from the ER causes the dissociation of Ca^{2+} from the EF hand domain of STIM1, which is accompanied by a substantial unfolding of the STIM amino terminus, leading to the multimerization and aggregation of STIM1 proteins through the exposure of several hydrophobic residues [123,135,141,146,147] (Fig. 1). Mutations that disrupt Ca^{2+} binding to the EF hand domain, or that destabilize the close interaction between the two EF-hand domains and the SAM domain, have been shown to result in constitutive STIM multimerization with subsequent activation of CRAC channels independent of ER Ca^{2+} levels [117,141,145,148–150].

In addition to its function as an ER Ca^{2+} sensor, STIM plays a key role in conveying Ca^{2+}-based signals from the ER to ORAI proteins [117,118,151]. The STIM carboxyl terminus contains three coiled-coil domains, of which two are sufficient for ORAI1 activation through the formation of a direct interaction with ORAI1, and thus constitute a minimal CRAC channel activation domain (CAD) [152–156]. A poly-basic lysine rich domain at the end of the carboxyl terminus apparently allows STIM recruitment to the plasma membrane [117,157].

STIM1 multimers then translocate to junctional ER sites that are in close association with the plasma membrane, and cluster into a discrete punctate pattern (also termed "puncta") [99,135,138–141,143,147,158,159]. ORAI1 is recruited to these puncta [135,138,152], and fluorescence resonance energy transfer (FRET) measurements between fluorescently tagged versions of STIM1 and ORAI1 indicate that these proteins are close enough to directly interact [159]. However, this interaction does not exclude the possibility that additional adaptor proteins might also be involved in further modulating STIM1-ORAI1 coupling. The STIM1/ORAI1 interaction appears to mediate opening of the ORAI channels [135,137,140,146,152,158], and, in T cells, was shown to occur rapidly [159]. The association is independent of channel activity or calcium influx, as was indicated by FRET measurements that showed an interaction between STIM1 and a mutant form of ORAI1 that eliminates Ca^{2+} entry through CRAC channels [135,137]. Furthermore, in T cells, the clusters containing STIM1 and ORAI1 have been shown to accumulate at the IS in a punctate pattern, resulting in increased local Ca^{2+} concentration [99,159,160] (Fig. 1). However, the clustering of STIM1 and ORAI1 at the contact surface, as well as their recruitment to the contact surface, do not depend on Ca^{2+} influx as they occur normally in cells transfected with non-functional mutants of ORAI1 [99,159]. Although clusters of STIM1 and ORAI1 at the contact surface were seen near the site of TCR engagement, these clusters were distinct from microclusters containing TCR and other signaling molecules [159].

These puncta were observed both in immune and non-immune cells, regardless of whether the cells were stimulated in a physiological (e.g. anti-TCR coated beads, superantigen pulsed B cells) or non-physiological (e.g. pharmacological depletion of ER with thapsigargin (TG), the Ca^{2+} ionophore ionomycin, IP_3, lectins) manner.

Barr et al. discovered that in T cells, in-addition to the puncta formation at the IS, STIM1 and ORAI1 also cluster in distinct cap-like structures that were localized to the distal pole of the cells, opposite to the contact surface [159]. It was further shown that in stimulated T cells, the caps were dynamic and sometimes translocated to a nascent IS. Based on these findings, the authors proposed that the caps might serve as a storage site for pre-assembled CRAC channels ready to be used at short notice, or that they could prevent CRAC channel activity outside the IS [159]. Thus, these caps should be important in sustaining Ca^{2+} influx by providing new STIM1-ORAI1 complexes, while avoiding inefficient Ca^{2+} signaling in areas outside of the IS.

5. Actin polymerization in immune cells

The cytoskeleton is deeply involved in a variety of cellular processes, including migration, adhesion, intracellular trafficking, cell polarity, cell division, endocytosis, and the formation of distinct cellular morphologies. These processes depend on the rapid assembly of filamentous actin (F-actin), which provides the basis for cellular structural remodeling and intracellular signal transduction [161,162]. The diversity and flexibility of cellular function are achieved by the formation of different actin structures, e.g. filopodia and lamellipodia for motility, podosomes for adhesion, and invadopodia for tissue invasion.

Spontaneous F-actin polymerization involves the formation of highly unstable actin dimer intermediates that are short-lived and rapidly dissociate, rendering spontaneous nucleation and elongation of new actin filaments kinetically unfavorable. Additionally, the vast majority of G-actin in eukaryotic cells is bound to profilin, which, along with actin severing and capping proteins, raise another obstacle to spontaneous nucleation [163]. These constraints make actin nucleation the rate-limiting step in the process of actin polymerization.

In order to support efficient actin polymerization, eukaryotic cells harness the activity of various actin elongation and nucleation proteins [164,165]. So far, three classes of nucleation proteins have been identified: the ARP2/3 complex, the formins, and the tandem-monomer-binding nucleators (Cordon-bleu (Cobl), Leiomodin (Lmod), and Spire) [166]. These proteins enable actin polymerization by initially stabilizing actin dimers or trimers, creating an actin "seed". Once a stable "seed" is formed, filament elongation continues at its fast-growing ('+' or 'barbed') end at a rate linearly proportional to the concentration of available actin monomers. The ARP2/3 complex facilitates the polymerization of branched F-actin, whereas the other two classes, i.e. the formins and tandem-monomer-binding nucleators, drive the

polymerization and elongation of linear, unbranched F-actin forms. Each class of nucleators uses a different mechanism to catalyze nucleation. ARP2/3 structurally mimics actin dimers that serve as a template for a third actin monomer to form a nucleus for actin polymerization [162,167]. Formins associate strongly with spontaneously formed actin dimers, and the tandem-monomer-binding nucleators bring together actin monomers through their clustered actin-binding motifs to form an actin nucleus [168]. Nevertheless, actin nucleation proteins such as ARP2/3 are inefficient nucleators by themselves and require other proteins, known as actin nucleation promoting factors (NPFs), to be efficiently activated [167]. There are currently eight known eukaryotic NPFs including the Wiskott–Aldrich syndrome protein (WASp), the WASp homologue, neuronal-WASp (N-WASp), WASp family Verprolin homologous protein (WAVE1-3), the Wiskott–Aldrich syndrome protein and SCAR homolog (WASH) [169], WASp homolog associated with actin membranes and microtubules (WHAMM) [170], and the junction mediating regulatory protein (JMY) [171]. The properties of these proteins and their specific functions have been extensively reviewed [172,173].

6. The interplay between the cytoskeletal networks and Ca^{2+} dynamics in T lymphocytes

The actin cytoskeleton and Ca^{2+} signaling present a complex molecular relationship in which each constituent is necessary for the persistence of the other (Fig. 2).

Actin filaments play an essential role in Ca^{2+} signaling throughout the various stages of T cell activation. TCR MC formation is highly dependent upon the actin cytoskeleton [20,23–26]. Since much of the signaling activity takes place within these MCs, disrupting actin filaments with either cytochalasin D or latrunculin A has been shown to inhibit Ca^{2+} signaling downstream of the TCR [26,174–176]. Additionally, the localization of many proteins involved in Ca^{2+} signaling depends on the cytoskeleton. Thus, T cells deficient in actin regulatory proteins such as Vav, WASp, WAVE2, Dynamin2 and HS1 often display impaired Ca^{2+} mobilization [16–19,21,22]. Furthermore, sustained Ca^{2+} signaling requires continued MC formation as well as continued actin polymerization [20]. Finally, F-actin is important for both the coalescence and internalization of TCR MCs followed by their degradation [20,24], illustrating the pivotal role of the actin cytoskeleton in signal termination, including that of Ca^{2+} signaling (Fig. 2).

Another aspect, Ca^{2+} directly regulates the activity of actin severing and capping proteins such as gelsolin [177]. Gelsolin is an actin binding protein, which can promote the assembly of monomers into filaments, as well as severing existing filaments. This protein is composed of six homologous domains, referred to as G1-G6 (from the amino terminus). Each domain contains a Ca^{2+} binding site. Gelsolin activity is regulated by Ca^{2+}. At low Ca^{2+} concentrations, the carboxyl terminus tail of the G6 domain masks actin binding sites on the amino terminus, and thus inhibits actin binding. Ca^{2+} binding to the G6 domain induces a conformational change that releases the tail domain and exposes the actin binding sites [178–182].

A Ca^{2+} increase in the cytoplasm is necessary for rapid cytoskeleton dependent polarization, which involves F-actin rearrangement [26,183,184] and MTOC reorientation [14,15] towards the region of T cell-APC interaction, eventually leading to the formation of the IS, as well as to the organization and maturation of the contact site into a symmetric long lasting synapse (Fig. 2).

In the following sections, we will focus on current evidence describing how the actin and microtubule cytoskeletal networks feedback to the signaling machinery by enabling the sustained intracellular Ca^{2+} signaling required for the T cell immune response.

6.1. The role of the cytoskeleton in STIM1-ORAI1 dynamics induced by TCR stimulation

STIM1 associates with microtubules through an interaction with the microtubule plus-end tracking proteins, EB1 and EB3 [185,186]. Treatment of T cells with colchicine, which induces depolymerization of microtubules, has little effect on Ca^{2+} influx and does not affect the STIM1 and ORAI1 interaction or their clustering at the distal side of the cell. However, while STIM1 and ORAI1 still cluster farthest from the contact surface under these conditions, they do not form normal cap structures. Disruption of the actin network by latrunculin has a

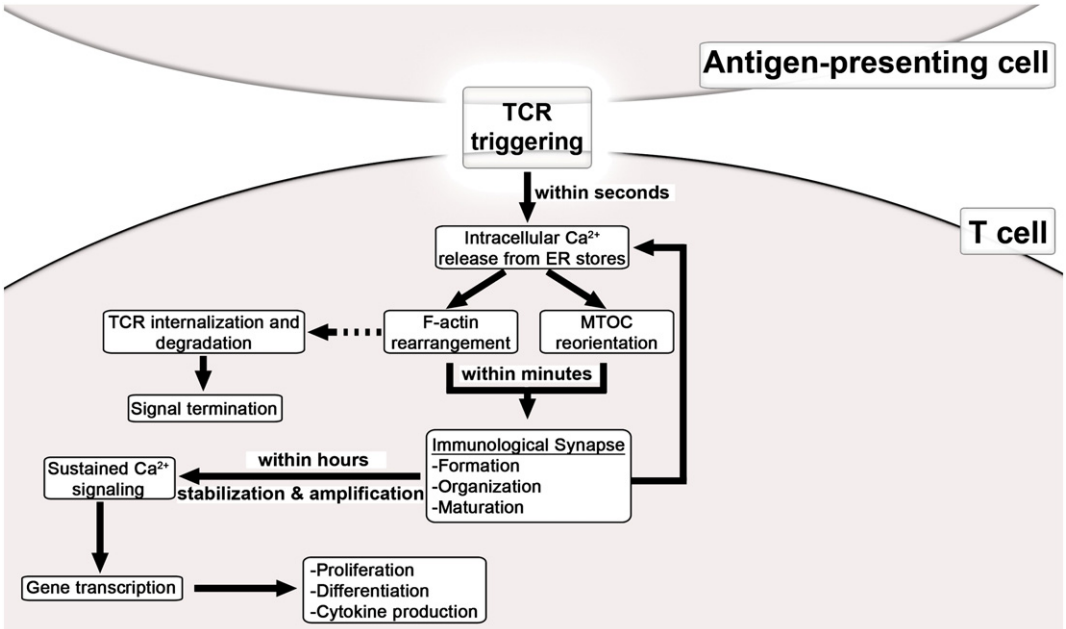

Fig. 2. A positive feedback loop between cytoskeletal networks and Ca^{2+} mobilization. TCR triggering leads, within seconds, to intracellular Ca^{2+} release from ER stores through the formation of TCR MCs. Once enough Ca^{2+} has entered the cell, it stabilizes the IS by promoting rapid cytoskeletal polarization over a period of a few minutes. A mature long lasting synapse, in turn, allows increased Ca^{2+} entry, through cytoskeleton dependent transport processes. Thus, the cytoskeleton plays a significant role in sustaining and amplifying TCR induced Ca^{2+} signals required for long term activation of T cells, including gene transcription, cytokine production and T cell proliferation.

profound effect on cap formation [159]. Thus, although the cytoskeleton is not required for the puncta formation of STIM1 and ORAI1 in T cells [139,140], both the microtubules and actin play an essential role in cap formation. Studies investigating the exact mechanism by which the cytoskeleton regulates cap formation would provide insight to the potential role of caps in Ca^{2+} signaling extension.

Recently, it was shown that CRAC channels function normally in actin immobilized T cells (i.e. under conditions in which actin turnover is blocked) [79], which is in agreement with another study demonstrating that in T cells, actin rearrangement is dispensable for CRAC channel activity [187]. Despite of these observations, a study by Nolz et al. showed that WAVE2-suppressed T cells exhibit reduced influx of Ca^{2+} following TCR ligation and also following TG stimulation. The inability of TG to rescue Ca^{2+} influx in WAVE2-suppressed T cells indicates a defect downstream of store depletion, suggesting that actin-regulating proteins can modulate Ca^{2+} influx through CRAC channels [19].

Another possible explanation for the observed reduction in Ca^{2+} influx in WAVE2 knocked down cells is a defect in the localization of signaling components that are involved in the Ca^{2+} signaling pathway downstream of store depletion. Since it has not been shown by patch clamp experiments that WAVE2 directly affects CRAC channels, it is possible that other modulators of Ca^{2+} entry, including mitochondria and PMCA (discussed in detail in Section 6.2), are affected by WAVE2. Another possibility, which should be considered, is that WAVE2 might regulate Ca^{2+} influx independently of its actin nucleation activity.

So far, the exact mechanism by which WAVE2 interferes with coupling of ER store release to CRAC channel opening remains elusive. Direct studies are required to determine the mechanism by which this protein participates in CRAC channel gating, and whether this involves its effect on the actin cytoskeleton.

Thus, it is now clear that there are additional steps that lie between store depletion and CRAC channel opening. These steps might involve actin regulatory proteins, e.g., WAVE2. As these steps are crucial for the proper functioning of T cells, it is of a great importance to explore them in greater detail.

6.2. Cytoskeleton-mediated translocation of mitochondria following T cell stimulation enables efficient CRAC channel activation

Mitochondria are essential for Ca^{2+} homeostasis in fundamental cellular functions such as metabolism and signaling in a large variety of cell types [188–193]. Interestingly, the ability of mitochondria to regulate Ca^{2+} signaling depends on their sub-cellular localization [190,194]. An electrochemical gradient generated during oxidative phosphorylation within the mitochondria enables the transport of free Ca^{2+} through the mitochondrial outer membrane. The Ca^{2+} is then taken up into the mitochondrial matrix by a calcium uniporter located on the inner mitochondrial membrane. In the matrix, Ca^{2+} is buffered by Ca^{2+} phosphate and subsequently extruded either by a sodium–calcium exchanger or by calcium-induced calcium-release pathways [195,196].

In T cells, mitochondria were found to be involved in maintaining Ca^{2+} influx across the plasma membrane through a tight functional interaction with CRAC channels [1,97,116,194,197–199] (Fig. 1). Using total internal reflection fluorescence microscopy (TIRFM), Quintana et al. showed that following IS formation, mitochondria are actively translocated towards the IS and are localized around 200 nm away from CRAC channels [160,194,200] (Fig. 1). This translocation is mediated by the cytoskeleton [194,201–203], and was found to be under control of co-stimulatory signals through the TCR, and chemokine receptor-dependent LFA-1 integrin activation [204].

Treatment with nocodazole, a microtubule inhibitor, significantly decreases mitochondrial translocation to the IS and greatly reduces prolonged Ca^{2+} signals in T cells stimulated with TG, which bypasses TCR signaling and IS formation. This indicates the importance of microtubule-dependent mitochondrial translocation toward the plasma membrane in sustaining Ca^{2+} signals [205]. The same results were achieved in cells pretreated with latrunculin B, an inhibitor of actin polymerization, in response to TG and stimulation with immobilized anti-CD3, which induces IS formation in addition to maximal store depletion [194]. However, in this case, it is difficult to determine whether or not the observed reduction in Ca^{2+} signals is due to the direct inhibition of mitochondrial transport, since TCR engagement and IS formation are also highly dependent upon actin polymerization. Intriguingly, treatment with nocodazole in cells stimulated with TG and immobilized anti-CD3, or treatment with latrunculin B in cells stimulated with TG alone, does not alter mitochondrial movement or Ca^{2+} signals [194]. In agreement with these findings, treatment with cytochalasin D, another actin polymerization inhibitor, or jasplakinolide, an actin stabilization agent, does not affect cytosolic Ca^{2+} influx in T cells following TG stimulation [187]. Thus, although both the microtubule and the actin cytoskeleton networks are involved in mitochondrial transport, they mediate this effect by different and independent mechanisms. Whereas the actin cytoskeleton mediates mitochondrial translocation following IS formation by an unknown mechanism, this translocation seems to be microtubule-based as long as the IS has not been formed.

Whereas a small cytosolic Ca^{2+} rise is required for mitochondrial motility [198], high Ca^{2+} levels, such as at sites of Ca^{2+} elevations near the opening of CRAC channels, reduce the motility of mitochondria [202,205,206], and would thus ensure the anchoring of mitochondria in the vicinity of CRAC channels (Fig. 1).

Such close proximity between mitochondria and CRAC channels allows mitochondria, which function as a highly dynamic Ca^{2+} buffer, to efficiently decrease the generation of local Ca^{2+} microdomains close to CRAC channels [199,207] (Fig. 1). Interestingly, Ca^{2+} ions themselves have been shown to inactivate CRAC channels in different ways, including both fast and slow inactivation pathways [5,10,11].

Mitochondrial relocalization during IS formation thus serves to suppress the Ca^{2+}-dependent inactivation of CRAC channels, and thereby prolongs CRAC activity for an extended period, enabling the sustained Ca^{2+} entry required for Ca^{2+} dependent activation of transcription factors and proper T cell function [160,194,204] (Fig. 1). Additionally, the enhanced entry of Ca^{2+} ions can be used to immobilize additional mitochondria in the vicinity, providing a positive Ca^{2+}-dependent feedback loop that would further extend Ca^{2+} signaling [208]. Not surprisingly, inhibition of mitochondrial Ca^{2+} uptake in T cells, greatly diminishes the cytosolic Ca^{2+} signal [190]. It was also found that mitochondria at the IS take up significantly more Ca^{2+} than mitochondria more distant from the IS [194].

Not only do mitochondria take up Ca^{2+}, but the imported Ca^{2+} is then released away from the CRAC channels to other locations in the cytosol. In this manner, mitochondria are able to propagate Ca^{2+} signals inside the cell (Fig. 1). Indeed, it was discovered that mitochondrial recruitment to the IS extensively enhanced Ca^{2+} signals [160,194,204].

In T cells, transient Ca^{2+} entry is not sufficient to activate transcriptional pathways, which require continuous Ca^{2+} entry through CRAC channels [209]. As mentioned in Section 3, NFAT transcriptional activity requires sustained elevation of intracellular Ca^{2+} levels. Contento et al. [204] further found that NFAT nuclear translocation is significantly higher in T cells in which mitochondria are recruited to the IS, than in cells in which mitochondria are not recruited, indicating that by sustaining Ca^{2+} influx, mitochondria recruited to the IS support the nuclear translocation of NFAT, and thus facilitate T cell activation.

Because of the physical interaction between STIM1 and ORAI1, which is necessary for the CRAC channel activity, mitochondria cannot directly reach the channels, but are localized around 200 nm away from them. Thus, mitochondria cannot independently regulate

channel activity [160]. It is therefore reasonable to assume that the mitochondrial-dependent Ca^{2+} microdomains that are formed around the channels may regulate the function of Ca^{2+} binding proteins such as calmodulin, which binds to the polybasic domain of STIM [210], and CRACR2A, which participates in the formation and stability of STIM–ORAI1 complex [211]. Both proteins may thus help mitochondria influence CRAC channel activity. Another means by which mitochondria prevent Ca^{2+}-dependent inactivation of CRAC channels is through the production of ATP, which serves as an effective Ca^{2+} chelator due to its rapid reaction with Ca^{2+} [197,212,213].

Mitochondria at the IS also co-localize with the PMCA [160]. Since PMCA is a major Ca^{2+} extrusion pathway in T cells [114], and thus Ca^{2+} ions transported through the PMCA out of the cell could be lost for signaling, this co-localization is of a great importance as it allows mitochondria to rapidly take up the inflowing Ca^{2+}, and thereby inhibit Ca^{2+}-dependent PMCA upregulation, resulting in decreased Ca^{2+} clearance by PMCA [160] (Fig. 1). Thus, mitochondria prevent inefficient Ca^{2+} recycling between CRAC channels and PMCA. However, in this context, the role of mitochondria in supplying ATP should also be considered, as Ca^{2+} pumping by PMCA is an ATP-dependent process, and thus, mitochondria can favor Ca^{2+} efflux during certain periods of signaling by regulating the local energy balance.

It is tempting to speculate that by supplying ATP to the PMCA, mitochondria might play a significant role in three processes: (i) Adjusting the efflux rate through PMCA to match influx through CRAC channels. This process may be important for proper T cell activation, as persistently elevated levels of intracellular Ca^{2+} and the subsequent persistent nuclear localization of NFAT have been associated with T cell anergy [214,215]. (ii) Inducing different Ca^{2+} signaling pathways. Since Ca^{2+} is a versatile messenger which can act through multiple pathways, depending on the magnitude and duration of changes in the Ca^{2+} concentration [90,93], mitochondria could support the different activation requirements of distinct transcriptional pathways. (iii) Finally, mitochondria may play a role in Ca^{2+} signal termination, and by extension, in T cell signaling downregulation. These assumptions should be substantiated by additional studies.

The relocalization of the PMCA to the plasma membrane was suggested to be regulated by the actin cytoskeleton, as treatment with cytochalasin D blocked PMCA translocation [216]. Unexpectedly, however, it was found that disruption of actin polymerization, with either cytochalasin D or latrunculin B, results in prolonged intracellular Ca^{2+} elevation, and subsequently, in augmented cytokine production, in response to TCR ligation or TG stimulation, which appears to contradict the results described above. The reason for this discrepancy is not yet known, and thus, further work is essential for characterizing the precise contribution of the actin cytoskeleton to the interplay between mitochondria and PMCA, and should provide additional insights into the role of the cytoskeleton in sustaining Ca^{2+} signaling.

Beyond acting as a transport mechanism during IS formation, the cytoskeleton leads to a dramatic shape change of the cell and to cell polarization through rapid cytoskeletal rearrangements, enabling the redistribution of many receptors, signaling molecules, and organelles [207]. This polarization allows the close apposition between mitochondria and CRAC channels, enabling mitochondria to rapidly take up large amounts of inflowing Ca^{2+}, thereby avoiding formation of high Ca^{2+} microdomains next to the channels, which would prevent the slow Ca^{2+} dependent inactivation of CRAC channels [160,200] (Fig. 1).

Although mitochondrial translocation toward the plasma membrane is observed in response to non-physiological stimulation [205], mitochondria are detected within 200 nm of CRAC channels only after formation of the IS [160,200] (Fig. 1). Importantly, Ca^{2+} increase was found to be more sustained after physiological stimulation which induces IS formation, rather than after non-physiological stimulation, including TG, which does not induce IS formation but maximally activates CRAC channels. This sustained Ca^{2+} rise induced by the IS was completely abolished by inhibition of mitochondrial Ca^{2+} uptake [194]. Furthermore, nano-scale redistribution of mitochondria, PMCA and CRAC channels can only be induced during the formation of the IS [160] (Fig. 1). Thus, cytoskeleton dependent morphological changes and organelle redistribution, resulting from cell polarization induced by the IS, are essential for the maintenance of Ca^{2+} signaling, and therefore ensure the efficient activation of T cells.

Altogether, these data suggest that by acting as a transport mechanism and by changing the relative distances between organelles through their spatial and temporal relocalization, the cytoskeleton provides the foundation for a positive Ca^{2+}-dependent feedback loop, and maximizes the efficiency of calcium influx through CRAC channels, needed for the amplification of TCR-dependent Ca^{2+} signals, which are required for an appropriate T cell response (Fig. 1).

7. Perspective

In this review, we have described the reciprocal interdependence between Ca^{2+} signaling and cytoskeletal rearrangements. However, detailed mechanistic characterization of the cross-talk between Ca^{2+} signaling and the cytoskeletal network in T cells is still lacking. Key questions remain to be answered, including: What are the key factors that link Ca^{2+} and the cytoskeleton? How does the cytoskeleton regulate cap formation? What is the mechanism that mediates the transition of mitochondria from the microtubule cytoskeletal network to the actin cytoskeletal network? Defective Ca^{2+} signaling in T cells that are deficient in specific cytoskeleton regulators may hint at their role in this cross-talk. In this regard, are the actin regulatory proteins, required for cytoskeletal rearrangement processes following TCR ligation, also involved in the translocation and reorganization of the mitochondria and the PMCA? To deepen our understanding in this area, studies using a combined approach of tracking Ca^{2+} flux together with examining the spatial and temporal redistribution of Ca^{2+} sources and sinks as compared with the cytoskeleton and its regulators, are required. Use of specific inhibitors of nucleation factors and/or RNAi to interfere with cytoskeletal regulators, in combination with live imaging of Ca^{2+} flux and cytoskeleton dynamics are likely to resolve the complexity of such a reciprocal interdependence.

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