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Immunological disorders: Regulation of Ca^{2+} signaling in T lymphocytes

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Abstract

Engagement of T cell receptors (TCRs) with cognate antigens triggers cascades of signaling pathways in helper T cells. TCR signaling is essential for the effector function of helper T cells including proliferation, differentiation and cytokine production. It also modulates effector T cell fate by inducing cell death, anergy (non-responsiveness), exhaustion and generation of regulatory T cells. One of the main axes of TCR signaling is the Ca^{2+}-calcineurin-nuclear factor of activated T cells (NFAT) signaling pathway. Stimulation of TCRs triggers depletion of intracellular Ca^{2+} store and in turn, activates store-operated Ca^{2+} entry (SOCE) to raise the intracellular Ca^{2+} concentration. SOCE in T cells is mediated by the Ca^{2+} release-activated Ca^{2+} (CRAC) channels, which have been very well characterized in terms of their electrophysiological properties. Identification of STIM1 as a sensor to detect depletion of the endoplasmic reticulum (ER) Ca^{2+} store and Orai1 as the pore subunit of CRAC channels has dramatically advanced our understanding of the regulatory mechanism of Ca^{2+} signaling in T cells. In this review, we discuss our current understanding of Ca^{2+} signaling in T cells with specific focus on the mechanism of CRAC channel activation and regulation via protein interactions. In addition, we will discuss the role of CRAC channels in effector T cells based on the analyses of genetically modified animal models.

Keywords

T cell receptor signaling; CRAC channels; Orai and STIM proteins; effector T cells; cytokine production; differentiation of T cells

Introduction

Ca^{2+} ion is ubiquitously utilized as a second messenger to regulate diverse cellular functions. However, in spite of its broad role, Ca^{2+} signaling has specificity to activate distinct downstream signaling pathways depending on the types of ligands and agonists. Each cell type including T cells expresses a unique blend of various Ca^{2+} channels

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or transporters to mediate Ca^{2+} signaling. Under resting conditions, cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]$) in T cells is in the range of ~100 nM while that in the endoplasmic reticulum (ER), which serves as an intracellular Ca^{2+} store, is much higher (~400 μM). Extracellular $[Ca^{2+}]$ (e.g., blood) reaches almost 2 mM, establishing a huge $[Ca^{2+}]$ gradient between the extracellular milieu, Ca^{2+} store, and the cytoplasm. Therefore, maintaining Ca^{2+} homeostasis in the resting state requires dynamic Ca^{2+} entry and exclusion across the membranes. After TCR stimulation, Ca^{2+} entry via store-operated Ca^{2+} (SOC) channels is a predominant mechanism to increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in T cells (Cahalan et al., 2009; Hogan et al., 2010; Lewis, 2011; Putney, 2009; Srikanth et al., 2013b). SOC channels were so named because they are activated by depletion of intracellular Ca^{2+} stores (Putney, 2009, 1986). The Ca^{2+} release-activated Ca^{2+} (CRAC) channel is a prototype and specialized class of SOC channel in immune cells. Increased Ca^{2+} ions can affect many signaling pathways, for example, via directly binding to anionic phospholipids or activation of Ca^{2+}-sensing signaling amplifiers including calmodulin (CaM), which in turn activate a large number of protein kinases/phosphatases to affect gene expression. Because the volume of ER in T lymphocytes is much smaller than that in other cell types, SOCE via CRAC channels is particularly important for activation of the NFAT (nuclear factor of activated T cells) family of transcription factors, which require sustained levels of increased $[Ca^{2+}]_i$. In the current review, we will focus on our current understanding of the regulation of Ca^{2+} signaling in T cells and phenotypes of animal models lacking the key components of CRAC channels, which provide insights into their physiological roles in the immune system.

Signaling pathways activated by TCR stimulation

Upon pathogen infection, specialized innate (e.g. dendritic cells or macrophages) and adaptive immune cells (e.g. B cells) present foreign antigens on their surface together with major histocompatibility complex (MHC) class II molecules to activate helper T cells. Interactions between TCRs and foreign antigens presented by MHC class II molecules play an important role in T helper cell functions such as proliferation, differentiation into effector and memory cells, and massive cytokine production after being recruited into the affected sites of inflammation. In addition, interactions between self-peptides and TCRs are important for T cell development in the thymus, homeostasis, and pathological onset of autoimmune diseases (Sprent et al., 2011). Thus, understanding of TCR signaling is crucial for development of therapy to treat patients with suboptimal immune responses (e.g., immune deficiency), lymphoproliferative disorder, and self (or microbiota)-reactive T cell-mediated autoimmunity (e.g., type I diabetes, rheumatoid arthritis, multiple sclerosis, psoriasis and inflammatory bowel disease).

Antigen engagement of T cell receptor triggers a cascade of tyrosine phosphorylation events initiated by a co-receptor CD4 that interacts with lymphocyte-specific protein tyrosine kinase (LCK) through its intracellular domain (Fig. 1a). Antigen-loaded MHC class II molecules simultaneously interact with the TCR-CD3 (γ-ε, ε-δ and ζ-ζ chains) complex and the LCK-associated co-receptor CD4. LCK then phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3 ζ chain, which triggers the recruitment of zeta chain-associated protein kinase 70 (ZAP70). ZAP70 phosphorylates membrane-associated scaffold molecules, linker for activation of T cells (LAT) and SH2 domain-

containing leukocyte protein of 76 kDa (SLP76) (Balagopalan et al., 2010; Samelson, 2011; Wang et al., 2010). Together with LAT, SLP76 recruits a guanine nucleotide exchange factor (GEF) and signaling adaptor molecule, Vav1 and an enzyme, phospholipase C (PLC)-γ1. Vav1 accumulates at the immunological synapse (IS) and recruits small G proteins such as Rac1 and CDC42 (cell division control protein 42 homolog) to activate the c-Jun N-terminal kinase (JNK) and p38 MAPK (mitogen-activated protein kinase) pathways, leading to activation of AP1 transcription factors (Tybulewicz, 2005). Recently, we showed that Vav1 also interacts with a large G protein, CRACR2A to activate the JNK signaling pathway (see below, (Srikanth et al., 2016a)). Therefore, Vav1 acts as a signaling hub to recruit small and large G proteins to activate downstream JNK and p38 MAPK pathways. PLCγ1 recruited into the LAT/SLP76 complex hydrolyzes plasma membrane-localized phosphatidylinositol 4, 5-bisphosphate (PIP_2) into second messengers, inositol trisphosphate ($InsP_3$) and diacyl glycerol (DAG), leading to activation of the Ca^{2+}-NFAT, NK-κB and ERK (extracellular signal-regulated kinase)-MAPK pathways. DAG predominantly activates NF-κB signaling pathway via activation of protein kinase C-theta (PKCθ) (Coudronniere et al., 2000; Lin et al., 2000; Sun et al., 2000). PKCθ is required to stimulate the BCM complex consisting of Bcl-10/Carma 1/MALT1, NF-κB-inducible kinase (NIK), and inhibitor of NF-κB (IκB) kinase (IKK) that eventually phosphorylates IκB (Fig. 1a) (Muller et al., 2010; Smith-Garvin et al., 2009; Sun, 2012). Phosphorylation of IκB leads to its degradation, allowing for nuclear translocation of NF-κB transcription factors. DAG also activates Ras-mediated signaling pathway via activation of Ras guanine nucleotide releasing protein 1 (RasGRP1), which mediates phosphorylation-induced activation of AP-1 (Fos-Jun) transcription factors mediated by kinases, dual specificity mitogen-activated protein kinase kinase (MEK) 1/2 and ERKs. The other product of PLCγ1 enzymatic activity, $InsP_3$, binds to the $InsP_3$ receptor ($InsP_3R$) on the ER membrane and releases Ca^{2+} from the ER into the cytoplasm and this store depletion leads to activation of CRAC channels on the plasma membrane. Activation of both the Ca^{2+} and MAPK signaling pathways are essential for differentiation and cytokine production of helper T cells and dysregulation of these pathways result in immune deficiency or autoimmune disorders in humans and mice (Constant et al., 1997; Kyriakis et al., 2012; Zhu et al., 2010).

The Ca^{2+}-calcineurin-NFAT pathway in T cells

One of the most studied Ca^{2+}-dependent signaling pathways in T cells is the calmodulin/calcineurin-NFAT pathway. Calcineurin is a Ca^{2+}-calmodulin complex-dependent serine/threonine protein phosphatase, consisting of a catalytic subunit, calcineurin A (CnAα, CnAβ, and CnAγ) and a regulatory subunit calcineurin B (CnB1 and CnB2). Upon increase of $[Ca^{2+}]_i$ via the CRAC channels, calmodulin binds Ca^{2+} and forms a complex with calcineurin, which in turn dephosphorylates the heavily-phosphorylated, cytoplasmic NFAT. NFAT consists of four homologous NFAT1 (NFATc2), NFAT2 (NFATc1), NFAT3 (NFATc4), and NFAT4 (NFATc3) (Hogan et al., 2003; Macian, 2005; Serfling et al., 2006; Srikanth et al., 2013b; Wu et al., 2007). Most of the NFAT family members are expressed in lymphocytes, however NFAT1 is predominantly expressed in naïve T cells and its short-term dephosphorylation after stimulation depends on the levels of Ca^{2+} entry. Expression levels of a short isoform of NFAT2 (NFATc1), NFAT2/αA is also Ca^{2+}-dependent (Serfling et

al., 2012). NFAT2/αA plays a more positive role in T cell activation than other NFAT family members by supporting proliferation and protecting T cells against cell death upon stimulation. Dephosphorylation of NFAT exposes its nuclear localization sequence (NLS) and induces its translocation from the cytoplasm to the nucleus (Gwack et al., 2007a; Hogan et al., 2003). Nuclear NFAT forms a multimeric protein complex with itself or with other general or lineage-specific transcription factors (e.g. AP-1) to induce gene transcription involved in cytokine production, cell proliferation, growth arrest, or cell death, depending on the amplitude and duration of $[Ca^{2+}]_i$ elevation (Kim et al., 2011; Macian, 2005; Macian et al., 2002).

NFAT proteins contain an N-terminal transactivation domain (TAD-N), a regulatory domain, a highly conserved DNA-binding domain (Rel-homology domain, RHD) and a C-terminal transcription activation domain (TAD-C) (Fig. 1b). NFAT proteins all bind to a similar DNA sequence containing 5'-(A/T)GGAAA-3' due to high conservancy within their DNA-binding domains, and thus are expected to have functional redundancy (Hogan et al., 2003). TADs are least conserved and this somehow explains distinct roles of each NFAT member because these domains can interact with different transcriptional co-activators (Mognol et al., 2016). NFATs are intrinsically localized in the cytoplasm, which is actively maintained by multiple kinases in the cytoplasm and the nucleus. The regulatory domain, which is moderately conserved among NFAT proteins, contains multiple serine-rich regions (SRRs) and Ser-Pro-X-X repeat motifs (SPs) that are phosphorylated by NFAT kinases including casein kinase I (CK1), glycogen synthase kinase 3 (GSK3), and dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK) family (Gwack et al., 2007a; Wu et al., 2007). CK1 phosphorylates the SRR-1 region of NFAT and functions as both, a maintenance kinase that keeps NFAT in the cytoplasm and an export kinase from the nucleus. GSK-3 functions as an export kinase and phosphorylates the SP-2 motif of NFAT1 and both the SP-2 and SP-3 motifs of NFAT2, and its activity is suppressed by Akt, a kinase activated in response to diverse signaling pathways in different cell types and by CD28 co-stimulatory signal (Gwack et al., 2007a). DYRKs phosphorylate the SP-3 motif of NFAT, thereby facilitating CK1- and GSK3-mediated phosphorylation of the SRR-1 and SP-2 motifs, respectively. Cytoplasmic DYRK2 serves as the maintenance kinase while nuclear DYRK1A serves as the export kinase. The substrate sites for GSK3 in NFAT are created after previous phosphorylation by a "priming" kinase that can be either protein kinase A (PKA) or DYRK1A (Arron et al., 2006; Gwack et al., 2006). Under resting conditions, heavily phosphorylated NFAT proteins exist in a complex with the non-coding RNA NRON (noncoding [RNA] repressor of NFAT). NRON creates a platform for RNA-protein scaffold complexes containing NFAT, NFAT kinases [e.g. CK1, GSK3, DYRK, and leucine-rich repeat kinase 2 (LRRK2)], IQ motif-containing GTPase activating protein (IQGAP), and CaM to facilitate phosphorylation/dephosphorylation events (Liu et al., 2011; Sharma et al., 2011; Willingham et al., 2005). The regulatory domain of NFAT also contains a docking site for calcineurin, with a highly conserved consensus sequence Pro-X-Ile-X-Ile-Thr (in which X can be any amino acid) (Aramburu et al., 1999). Upon TCR stimulation-induced increase in $[Ca^{2+}]_i$, Ca^{2+}-bound CaM activates calcineurin, which dephosphorylates multiple phosphoserines in the SRR and SP motifs of NFAT regardless of its distance from the calcineurin-binding site, exposing its NLS and facilitating nuclear translocation.

In addition to the Ca^{2+}-calcineurin-NFAT pathway, increased $[Ca^{2+}]_i$ also plays crucial role in activation of the NF-κB and ERK MAPK signaling pathways. During formation of the BCM complex, Ca^{2+} has been implicated in phosphorylation via calmodulin kinase II (CaMKII), which is important for IκB degradation (Ishiguro et al., 2007; Ishiguro et al., 2006; Oruganti et al., 2011). In addition, Ca^{2+}-activated PKCα phosphorylates NF-κB transcription factors, which is important for nuclear translocation of these factors (Liu et al., 2016). Elevated $[Ca^{2+}]_i$ also regulates the Ras-AP1 signaling pathway by binding to EF-hand motifs of RasGRP1 (Mor et al., 2006). The Ras guanine nucleotide exchange factor (RasGEF) activity of RasGRP1 on the Golgi depends on both DAG and Ca^{2+} that eventually leads to activation of the AP-1 transcription factor. Therefore, Ca^{2+} signaling is integrated with other signaling pathways at the DNA response elements of NFAT, NF-κB and AP-1 transcription factors, resulting in cell proliferation and survival, cytokine gene expression, differentiation, or cell death depending on the intensity of diverse signaling pathways.

Specificity of Ca^{2+} signaling

Ca^{2+} signaling plays an important role in diverse cellular functions. However, numerous evidences suggest that Ca^{2+} can play a specialized role in activation of specific signaling pathways depending on the amplitude, oscillation frequency, and location of its entry. In physiological conditions, unlike treatment with ionophore or a blocker of SERCA (sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase), thapsigargin, T cells show Ca^{2+} oscillations after TCR engagement that is regulated by a balance between cytoplasmic and ER Ca^{2+} concentrations as well as the levels of Ca^{2+} entry and exclusion (Dolmetsch et al., 1994; Dolmetsch et al., 1998). NFAT, AP-1 and NF-κB transcription factors were shown to be optimally activated in response to different oscillation patterns of Ca^{2+} in T cells. Transient high Ca^{2+} spikes evoked activation of JNK and NF-κB, but not NFAT, whereas prolonged low increases in $[Ca^{2+}]_i$, was sufficient for activation of NFAT, but not JNK or NF-κB (Dolmetsch et al., 1997). It was also shown that $[Ca^{2+}]$ in the microdomains near the CRAC channels is more important for nuclear translocation of NFAT than global increase of Ca^{2+} emphasizing the importance of local Ca^{2+} concentration in T cells (Kar et al., 2011). STIM1 and Orai1 proteins are crucial components of the CRAC channel in T cells (see below). Upon antigen engagement of $CD4^+$ T cells, Orai1 and STIM1 translocate into the immunological synapse, a site of contact between the TCRs and antigen-loaded MHC class II molecules, suggesting that Ca^{2+} entry via CRAC channels occurs at specific locations in T cells (Barr et al., 2008; Lioudyno et al., 2008). The site of Orai1 and STIM1 clustering was expectedly proven to be the location of Ca^{2+} entry (Luik et al., 2006).

Recent studies have shown that Ca^{2+} ions were not important for recruitment of Orai1, STIM1, and TCRs into the immunological synapse at the initial phase, but played an essential role in actin re-organization, which was crucial for the stability of the immunological synapse (Hartzell et al., 2016; Lioudyno et al., 2008). In addition, increased local $[Ca^{2+}]$ at the immunological synapse is also important for sustaining CD3 phosphorylation by neutralizing negative charges of phospholipids and generating unfolded structure of the cytoplasmic domains of CD3 chains (Shi et al., 2013). Since the immunological synapse is the site for aggregation of signaling molecules including many tyrosine and serine/threonine kinases, it is possible that clustering of Orai1 and

STIM1 at the synapse is regulated by protein phosphorylation via these TCR signaling molecules, but there has been no experimental data to prove this yet. Together, these studies suggest that not only the amplitude of Ca^{2+} signaling, but also its pattern and location (e.g. oscillation frequency, sustained levels, and microdomains) can provide specificity to activate downstream signaling pathways.

Components of CRAC channels in T cells - Orai and STIM proteins

CRAC channels in T cells consist of pore subunits, Orai proteins and regulatory STIM proteins. Differently from many other Ca^{2+} channels, CRAC channels are primarily gated by a direct interaction between Orai and STIM proteins. Existence of CRAC channels in immune cells was identified by electrophysiological methods based on their unique biophysical characteristics (Hoth et al., 1992; Lewis et al., 1989). Later, genome-wide RNAi screens in *Drosophila* cells identified the *Drosophila* gene *olf186-F* (named *Drosophila* Orai) and its mammalian homologues Orai1, 2 and 3 as subunits of the CRAC channels (Feske et al., 2006; Gwack et al., 2007c; Vig et al., 2006; Zhang et al., 2006). Furthermore, a missense mutation of R91W was identified in the ORAI1 gene from severe combined immune deficiency (SCID) patients that lacked functional CRAC channels and expression of wild-type Orai1 recovered CRAC currents in patient cells (Feske et al., 2006). Prior to identification of Orai1, limited RNAi screen in *Drosophila* and HeLa cells identified STIM1, a Ca^{2+}-sensing protein localized predominantly in the endoplasmic reticulum (ER) as an important regulator of CRAC channel-mediated Ca^{2+} entry (Liou et al., 2005; Roos et al., 2005; Zhang et al., 2005).

Orai1 has four transmembrane segments (TM1-TM4) with its N and C terminus facing the cytoplasm. The TM1 of Orai1 has been shown to line the pore, and residues in the TM1 including R91, G98, V102, and E106 are important for Ca^{2+} selectivity and gating (Cahalan et al., 2009; Hogan et al., 2010; Lewis, 2011; McNally et al., 2012; Putney, 2009; Srikanth et al., 2013c; Zhang et al., 2011). The cytoplasmic N and C terminus of Orai1 mediates channel opening by interaction with STIM1 after store depletion. CRAC channels are also negatively regulated by excess Ca^{2+}, resulting in their Ca^{2+}-dependent inactivation (CDI) (Hoth et al., 1992, 1993; Zweifach et al., 1995). In addition to channel gating, mutational studies showed that all the cytoplasmic regions of Orai1 including the N terminus, the intracellular loop, and the C terminus are involved in CDI (Lee et al., 2009; Mullins et al., 2009; Srikanth et al., 2010b). Thus, intracellular domains of Orai1 are important not only for channel gating, but also for channel inactivation, to avoid deleterious consequences of excessive Ca^{2+} including cell death.

STIM family has two members, STIM1 and STIM2. STIM1 contains an N-terminal EF-hand that detects luminal ER [Ca^{2+}], a single transmembrane domain, and a long C-terminal cytoplasmic region (Fig. 2) [reviewed in (Soboloff et al., 2012)]. STIM1 plays a pivotal role in sensing ER [Ca^{2+}] and CRAC channel opening. Upon ER Ca^{2+} depletion, STIM1 loses bound Ca^{2+}, multimerizes, translocates to the plasma membrane-proximal ER, mediates clustering of Orai proteins, and stimulates Ca^{2+} entry (Liou et al., 2005; Roos et al., 2005; Zhang et al., 2005). STIM1 interacts with Orai1 via CRAC-activating domain (CAD)/ STIM1 Orai1 activating region (SOAR) (Muik et al., 2009; Park et al., 2009; Yuan et al.,

2009). The CAD/SOAR fragment of STIM1 (coiled coil domains 2 and 3) was shown to play a pivotal role in activation of Orai1 by direct binding to its cytoplasmic N and C terminus. Furthermore, the stoichiometry of STIM1 binding to Orai1 can affect the fast inactivation properties of CRAC channels, indicating that STIM1 is a bona fide subunit of CRAC channels (Scrimgeour et al., 2009). These and other studies showed that CRAC channel activation involves multiple steps including STIM1 oligomerization, co-clustering of Orai1 and STIM1 at the ER-plasma membrane junctions, and gating of Orai1 (Liou et al., 2007; Muik et al., 2009; Muik et al., 2008; Navarro-Borelly et al., 2008; Park et al., 2009; Yuan et al., 2009). STIM2 shares similar domain structure with STIM1, but its ER-luminal EF-hand motif has lower Ca^{2+} binding affinity than STIM1, based on translocation kinetics in response to varying ER [Ca^{2+}] (Brandman et al., 2007). Because STIM2 responds to subtle changes in ER [Ca^{2+}], it has been suggested to regulate basal [Ca^{2+}] (Brandman et al., 2007). STIM2 function in activation of Orai1 is not as strong as that of STIM1 and it also shows a slower kinetics in aggregation and translocation than STIM1 (reviewed in (Soboloff et al., 2012)). In T cells, the role of STIM2 is not obvious due to the primary function of STIM1 in SOCE, but it seems to play a supportive role for STIM1 function in SOCE and long-term activation of NFAT (Oh-Hora et al., 2008).

Cellular factors associating with Orai1 in T cells

In addition to Orai and STIM proteins, numerous auxiliary factors regulate activation and inactivation of CRAC channels by inducing conformational changes or stabilizing Orai1-STIM1 interaction. Interacting partners of Orai1 and their functions in general cell types have been recently summarized (Srikanth et al., 2012a, 2013a; Srikanth et al., 2013d). In theory, all of these interacting partners can act as regulators for Ca^{2+} signaling in T cells, but only the molecules whose functions have been validated in T cells will be discussed here.

An interacting partner of Orai1, CRAC channel regulator 2A (CRACR2A, EFCAB4B or FLJ33805) was identified from large-scale affinity protein purification using Orai1 as bait (Srikanth et al., 2010a). CRACR2A has two splice isoforms, CRACR2A-a (~80 KDa) and CRACR2A-c (45 KDa). The short isoform CRACR2A-c is cytoplasmic and forms a ternary complex with Orai1 and STIM1 to stabilize their interaction after store depletion. Accordingly, its depletion decreases STIM1 clustering at the ER-plasma membrane junctions and hence, SOCE. This interaction with Orai1 and STIM1 is $[Ca^{2+}]_i$-dependent, with low $[Ca^{2+}]_i$ favoring association and high $[Ca^{2+}]_i$ favoring its dissociation by sensing Ca^{2+} through its EF-hand motifs (Fig. 3a). The long isoform CRACR2A-a encodes a large Rab GTPase (Srikanth et al., 2016a; Srikanth et al., 2016b; Wilson et al., 2015). CRACR2A-a is localized to the proximal Golgi area and vesicles, and plays an important role in TCR signaling pathways including SOCE and JNK MAPK pathways (Srikanth et al., 2016a). While the regions for Ca^{2+} binding and interaction with Orai1 and STIM1 are conserved between the two isoforms, CRACR2A-a has an additional proline-rich domain (PRD) and a Rab GTPase domain in its C-terminus, and is abundantly expressed in lymphoid organs. CRACR2A-a is unique because it clearly distinguishes itself from small Rab GTPases (~20 KDa) due to its large size (~85 KDa) and presence of multiple functional domains (Srikanth et al., 2016a; Wilson et al., 2015). The Rab GTPase domain of CRACR2A-a contains conserved guanidine-binding residues that regulate its localization in a GTP/

GDP-binding and prenylation-dependent manner (Fig. 3b). GTP-bound and prenylated CRACR2A-a localizes within vesicles close to the *trans* Golgi network whereas GDP-bound or unprenylated CRACR2A-a is cytosolic and rapidly degraded. Prenylation of CRACR2A-a involves geranylgeranylation at an unconventional site (CCx, x; any amino acid) in the C terminus. Upon TCR stimulation CRACR2A-a translocates into the immunological synapse via interaction of its PRD domain with Vav1, to activate SOCE and both the Ca^{2+}-NFAT and the Jnk MAPK signaling pathways. CRACR2A-a also translocates into the ER-plasma membrane junctions via vesicle trafficking after passive ER Ca^{2+} store depletion and recovers SOCE in Jurkat T cells depleted for both the isoforms, similar to CRACR2A-c. Because CRACR2A-a retains the Orai-STIM interaction domain, one can assume that it supports SOCE by interacting with both Orai1 and STIM1, similar to CRACR2A-c.

The α-SNAP protein was also identified as a cytosolic factor that interacts with both Orai1 and STIM1 (Miao et al., 2013). Depletion of α-SNAP drastically decreased SOCE in various cell types including Jurkat T cells. The original function of α-SNAP is disassembly of the SNARE (NSF attachment protein receptor) complex, a cellular machinery used for vesicle fusion. Differently from its original function, α-SNAP, a predominantly cytoplasmic protein; physically interacts with the cytosolic CAD/SOAR domain of STIM1 and the C-terminal tail of Orai1. Through this interaction, α-SNAP regulates an active molecular rearrangement within Orai1-STIM1 clusters to obtain the STIM1/Orai1 ratio required for optimal activation of CRAC channels, without affecting the rate of STIM1 translocation into the ER-PM junctions. Accordingly, after store depletion, α-SNAP-depleted cells stably expressing Orai1 and STIM1 exhibited increase in density of Orai1 in clusters without altering STIM1 density, leading to a reduced ratio of STIM1/Orai1 in individual clusters, thereby reduced SOCE. A follow up study by the same group showed that α-SNAP deficiency induces formation of Orai1 oligomers with biophysical properties different from CRAC channels, including higher Na^+ permeability. Thus α-SNAP plays an important role in establishing a correct ratio between STIM1 and Orai1 by direct interaction with the two proteins (Li et al., 2016).

Cellular factors associating with STIM1 in T cells

Orai1 and STIM1 cluster at pre-existing junctions of the ER and the plasma membrane, a space of 10–25 nm (Varnai et al., 2007; Wu et al., 2006). In muscle cells, proteins localized to the junctions between the plasma membrane and ER/sarcoplasmic reticulum (SR) membrane form a structural foundation for Ca^{2+} dynamics essential for excitation-contraction coupling (Berridge et al., 2003; Carrasco et al., 2010). Various biochemical screening approaches have identified junctophilins, mitsugumins, sarcalumenin, junctin, and junctate as components of these junctions (Carrasco et al., 2010; Takeshima et al., 2000; Weisleder et al., 2008). Recent studies have shown that homologues and isoforms of these junctional proteins are also expressed in T cells. Srikanth et al identified the EF-hand containing protein, junctate as an interactor of STIM1 (Srikanth et al., 2012b). Junctate localization defined the sites of accumulation of CRAC channel components, since after store depletion, Orai1 and STIM1 accumulated at junctions that were already marked by junctate (Fig. 3c). The EF-hand motif of junctate senses ER Ca^{2+} depletion, which is important for efficient recruitment of STIM1. However, junctate alone cannot function as

a membrane-tethering factor because the cytoplasmic N terminus is very short and also does not contain any obvious plasma membrane-binding motif. In a recent study, Woo et al identified another junctional protein, junctophilin 4 (JP4) as an interacting partner of STIM1 (Woo et al., 2016). Junctophilin family consists of four genes JP1, JP2, JP3, and JP4 that are expressed in a tissue-specific manner and are known to form ER-plasma membrane junctions in excitable cells including skeletal muscle, cardiac and neuronal cells (Nishi et al., 2003; Takeshima et al., 2000). Junctophilins contain eight repeats of the membrane occupation and recognition nexus (MORN) motifs that bind to phospholipids in the N terminus and a C-terminal ER membrane-spanning transmembrane segment (Garbino et al., 2009; Takeshima et al., 2000). Depletion of JP4 inhibited STIM1 recruitment into the ER-plasma membrane junctions and significantly decreased SOCE. Biochemical analyses showed a direct interaction of JP4 cytoplasmic domain with coiled-coil 1 and 2 regions of STIM1. JP4 was also shown to interact with the N-terminal cytoplasmic region of junctate. Therefore, this study demonstrates that JP4-junctate complex is localized at the ER-plasma membrane junctions in T cells and synergistically recruits STIM1 into these junctions by direct interaction. When overexpressed, STIM1 alone is sufficient to establish the ER-plasma membrane junctions using its C-terminal poly-lysine tail. However, in a physiological condition when the concentration of STIM1 is limiting or when STIM1 is unable to bind membrane phospholipids (e.g., due to low [PIP_2] in the plasma membrane), its interaction with the juncate-JP4 complex can be important for efficient assembly of a functional CRAC channel complex at the ER-plasma membrane junctions.

Transmembrane protein 110 (TMEM110 or STIM-activating enhancer) was identified as a positive regulator of SOCE using biotin-labeled protein purification and a genome-wide RNAi screen, respectively (Jing et al., 2015; Quintana et al., 2015). TMEM110 is a multi-pass ER-resident protein with its N and C termini facing the cytoplasm (Fig. 3c). Jiang et al showed that TMEM110 interacted with the coiled-coil 1 region of STIM1 and induced its active conformation to interact with Orai1 (Jing et al., 2015). The coiled-coil 1 region of STIM1 contains an acidic amino acid motif that binds to the positively charged residues within Orai1-interacting CAD/SOAR fragment, blocking its interaction with Orai1 in an auto-inhibitory manner. Interaction of TMEM110 with the coiled coil 1 region of STIM1 facilitated the release of this auto-inhibition. Furthermore, this study showed that depletion of TMEM110 had a modest influence on the frequency of the ER-plasma membrane junctions with 8–12% decrease in cortical ER. Another study also identified TMEM110 as an important regulator of SOCE using an siRNA screen. This study showed that siRNA-mediated depletion of TMEM110 significantly reduced the density of ER-plasma membrane junctions by >60% in HeLa/HEK293 cells, both under resting conditions and after store depletion. Importantly, artificial expansion of the junctions by overexpression of a yeast junctional protein lst2, which is unlikely to affect STIM1 auto-inhibition, significantly rescued STIM1 translocation and SOCE. Therefore, this study concluded that TMEM110 is important for maintenance of the ER-PM junctions involved in SOCE in resting conditions and for dynamic remodeling of these junctions after store depletion. Both these studies show an important role of TMEM110 in STIM1 translocation and thereby SOCE in T cells, although further studies are required to validate whether this involves direct interaction with STIM1 or indirectly by regulating the number of ER-plasma membrane junctions.

An ER-resident protein SARAF was identified as an interacting partner of STIM1 and a negative regulator of SOCE, which facilitates the Ca^{2+}-dependent slow inactivation of CRAC channels (Palty et al., 2012). SARAF encodes a 339-amino acid protein containing single transmembrane segment (aa 173–195) with its N terminus facing the ER lumen (aa 1–172) and its C terminus facing the cytoplasm (aa 196–339) (Fig. 3c). SARAF contains positively charged residues, which may interact with the plasma membrane phospholipids and a serine/proline-rich domain in its C-terminal end. Depletion of SARAF increased intracellular Ca^{2+} concentration and enhanced SOCE after store depletion, whereas its overexpression showed an opposite effect. SARAF played a negative role in SOCE with multiple modes of action; i) interaction with the inactive form of STIM1 at the resting condition to stabilize its inactive conformation in the ER, ii) induction of Ca^{2+}-dependent inactivation of Orai1 channels after translocation to the ER-plasma membrane junctions together with STIM1, and iii) facilitating dissociation of clustered STIM1 proteins. Detailed structure-function studies identified a C-terminal inhibitory domain (CTID, aa 448–530) within STIM1 that regulates SARAF-STIM1 interaction (Jha et al., 2013). STIM1 CTID is located at the C-terminal region of the Orai1-interacting CAD/SOAR domain and interestingly, deletion of CTID from full-length STIM1 resulted in constitutively active Orai1 channels. CTID does not bind to SARAF directly, but mediates the interaction of SARAF with the CAD/SOAR region. Therefore, this study highlights the important role of STIM1 and SARAF in Ca^{2+}-dependent inactivation of Orai1. STIM1 also negatively regulates plasma membrane Ca^{2+} ATPase (PMCA) directly or indirectly via a novel 10-transmembrane segment-containing protein, POST (partner of STIM1, TMEM20) (Krapivinsky et al., 2011; Ritchie et al., 2012). These studies suggest that both stimulation of CRAC channels and inhibition of PMCA activity at the immunological synapse may be important for generation of sustained, local Ca^{2+} entry required for NFAT activation.

Ca^{2+} signaling in development of T cells in the thymus

In humans, loss of Orai1 or STIM1 function causes immune deficiency which is recapitulated in animal models lacking expression of these proteins. In human patients and mice lacking expression of Orai or STIM proteins, development of conventional TCRαβ+ T cells is normal. These results were unexpected because the intensity and duration of TCR signaling is important for positive and negative selection of T cells in the thymus and SOCE was expected to play a major role in these events. Although deficiency of any CRAC channel component does not influence development of conventional TCRαβ+ T cells, deficiency of both STIM1 and STIM2 impaired development of unconventional agonist-selected T cells, including regulatory T cells, invariant natural killer T (iNKT) cells and intestinal intraepithelial lymphocytes, which are thought to require strong and sustained TCR signals during development (Oh-Hora et al., 2013). STIM1 deficiency alone dramatically reduced development of iNKT cells, while that of regulatory T cells and intestinal intraepithelial cells was normal (Oh-Hora et al., 2008). These results suggest that SOCE is important for development of agonist-selected T cells, and especially iNKT cells require high levels of $[Ca^{2+}]_i$ for their development.

Instead of CRAC channels, development of T cells depends on other modes of Ca^{2+} signaling. Recent studies on voltage-activated Ca^{2+} channels (VOCCs) provide insights into

the role of Ca^{2+} signaling during T cell development and differentiation (Badou et al., 2013; Nohara et al., 2015). VOCCs are predominantly expressed in excitable cells and activated by action potentials. They are divided into L-type (Cav1.1–1.4), P/Q-type (Cav2.1), N-type (Cav2.2), R-type (Cav2.3) and T-types (Cav3.1, 3.2, and 3.3) (Christel and lee 2012). They consist of an α1 subunit and four auxiliary subunits of α2, δ, β, and γ. The α1 subunits line the pore of the Ca_V channels, whereas the β subunits are regulatory proteins that are important for assembly of the channel complex, correct plasma membrane targeting and stimulation of channel activity. Recently, it was found that deletion of only the β2 subunit profoundly influenced T cell development by decreasing the total number of thymocytes due to inhibition of the DN (double-negative)-to-DP (double positive) transition (Badou et al., 2013; Jha et al., 2015). Protein levels of Cav1.2 and Cav1.3 were decreased in β2 subunit-deficient thymocytes, suggesting that Cav1.2/1.3-β2 channels are important for thymic development of conventional T cells (Jha et al., 2015). Another study examined mice genetically deleted for expression of α1 subunit of $Ca_v1.4$ and observed subtle reduction in CD4 single positive cells in the thymus (Omilusik et al., 2011). Both the studies using mice deficient in $Ca_v1.4$ or the regulatory β subunits identified a positive role for Ca_v1 channels in T cell development.

Several questions still remain to be answered regarding the role of Ca^{2+} signaling during T cell development in the thymus. Ca_v channels are known to be activated by TCR stimulation, but Ca^{2+} entry via these channels is not as robust as those mediated by Orai channels. We do not understand how these moderate levels of Ca^{2+} entry mediated by Ca_v channels play an important role in T cell development while the robust SOCE observed with CRAC channels is dispensable for the same. Normal development of TCRαβ+ T cells in Orai1 KO or STIM1/STIM2 double KO animals, suggest that CRAC channels are not important during development stage. However, it is still possible that other Orai channels (e.g., Orai2 or Orai3) play a role in T cell development in a STIM-independent manner. In addition, the Ca^{2+} channel important for negative selection in the thymus has not been identified yet. Self-reactive T cells should have higher levels of Ca^{2+} signaling to trigger cell death (see below). Therefore, Ca^{2+} channels involved in negative selection are expected to have robust Ca^{2+} entry, but any defect in negative selection has not been identified in mice deficient for CRAC or Ca_v channel components. Therefore, Ca^{2+} channels that are important for each step of T cell development in the thymus remain to be uncovered in future work.

Orai and STIM proteins in the function of effector T cells

Naïve T cells undergo clonal selection, proliferation and differentiation into effector T cells at the central or peripheral lymphoid organs (e.g., spleen or lymph nodes). For differentiation into specific effector T cells, three conditions need to be met. Signals from TCRs and co-receptor (e.g., CD28) are essential for optimal differentiation. In addition, polarizing cytokines (e.g., IL-4, IL-6, or IL-12) are essential for determination of T cell fate such as differentiation into various effector T cells including Th1, Th2, Th9, Th17, Th22 and follicular T cells. Differentiation and effector functions of these cells are controlled by signature transcription factors, for example, T-bet for Th1, GATA3 for Th2, or RORγt for Th17 cells. After differentiation at the priming sites, effector helper T cells migrate

into the infected or inflamed tissues to produce cytokines that recruit and differentiate myeloid-lineage innate immune cells.

The most obvious defect in patients and mice with nonfunctional CRAC channels has been identified in various functions of effector T cells including differentiation and cytokine production. Patients with a point-mutation, R91W showed defect in cytokine production in T cells (Feske et al., 2006). In consistence, Orai1 KO or R93W (counterpart of human R91W mutation) knock-in mice also showed a reduction in cytokine production by $CD4^+$ and $CD8^+$ effector T cells (Gwack et al., 2008; McCarl et al., 2010). In addition to cytokine production, SOCE via Orai1 channels is essential for differentiation of effector T cells. Using a small molecule inhibitor of Orai1, compound 5D (N-[2,2,2-trichloro-1-(2-naphthylamino)ethyl]-2-furamide) identified from chemical library screen and Orai1-deficient T cells, it was shown that Orai1 plays a key role in differentiation of Th17 cells by induction of the NFAT-RORγt pathway (Kim et al., 2014). Inhibition or deficiency of Orai1 also decreased expression of T-bet and GATA3 under Th1- and Th2-polarizing conditions, respectively, but to a much lesser degree, suggesting a higher dependence on SOCE for Th17 differentiation. Decreased Th17 differentiation in Orai1-deficient T cells could be mimicked by treatment with cyclosporine A, a blocker of calcineurin, and this defect was rescued by expression of constitutively active NFAT at least partly, suggesting that the Orai1-NFAT pathway significantly contributes to expression of RORγt, and thus Th17 differentiation. Another report also validated the role of Orai1 in cytokine production in effector T cells using blockers and Orai1 knockout mice (Kaufmann et al., 2016). The major findings are consistent by demonstrating an important role of Orai1 in production of IL-17A and IFN-γ by effector T cells without affecting the function of regulatory T cells. However, there was discrepancy in the role of Orai1 in expression of T-bet and RORγt transcription factors, which could be due to differences in the residual Ca^{2+} entry between the two independent knockout mouse lines. In addition to Orai1, STIM1 deficiency also showed a pronounced reduction in SOCE and cytokine production in T cells resulting in resistance to experimental autoimmune encephalomyelitis (EAE) (Oh-Hora et al., 2008; Schuhmann et al.). On the contrary, mice deficient in STIM2, another member of the STIM family, showed a mild defect in SOCE and correspondingly, succumbed to EAE, albeit with less severe symptoms (Schuhmann et al.).

The genetic manipulation of Orai and STIM proteins provide opportunities to determine the outcomes of differential SOCE levels in the functions of effector T cells. For example, a progressive reduction in SOCE was observed in WT, $Orai1^{+/-}$ and $Orai1^{-/-}$ T cells (Fig. 4a). The residual SOCE in $Orai1^{-/-}$ T cells can be blocked by the widely used CRAC channel inhibitor 2-APB (2-aminoethoxydiphenyl borate), suggesting that the residual SOCE is likely mediated by Orai2 or Orai3 proteins (Srikanth S. and Gwack Y, unpublished). Hence, SOCE in $Orai1^{-/-}$ T cells can be further reduced by expression of a dominant negative mutant of Orai1, E106Q which can is likely to hetero-multimerize with Orai2 or Orai3 to further decrease SOCE (Gwack et al., 2007b; Kim et al., 2011). On the contrary, expression of a mutant of Orai1 that lacks fast inactivation ($Orai1^{MutA}$) can increase SOCE in $Orai1^{-/-}$ T cells (Srikanth et al., 2010c). These results provide ways to generate, in theory, five different gradients of SOCE in T cells, $Orai1^{-/-}$ T cells with expression of E106Q, $Orai1^{-/-}$, $Orai1^{+/-}$, $Orai1^{+/+}$ and $Orai1^{-/-}$ T cells with expression of MutA Orai1 (lowest to highest

[Ca^{2+}]) (Fig. 4a). These genetic modifications will provide useful tools to investigate various outcomes in T cell functions responding to different levels of SOCE.

There is no data regarding a direct comparison of SOCE between Orai1 and STIM1 KO T cells, but based on the published results, it is assumed that SOCE in $STIM1^{-/-}$ T cells should be much lower than that of $Orai1^{-/-}$ T cells. SOCE in $STIM1^{-/-}STIM2^{-/-}$ (DKO) T cells is close to basal levels, which is very similar with $Orai1^{-/-}$ T cells expressing $Orai1^{E106Q}$. Progressive decrease in SOCE observed in WT, $Orai1^{+/-}$, $Orai1^{-/-}$ or $Orai1^{E106Q}$-expressing cells closely correlated with cytokine expression and differentiation (as judged by the expression of key transcription factors and surface receptors) of effector T cells as depicted in Fig. 4b. There is a general notion that robust Ca^{2+} signaling is needed for T cell proliferation, based on the observation that absence or chelation of extracellular Ca^{2+} drastically decreases T cell proliferation. However, analysis of Orai and STIM knockout T cells reaches a different conclusion, with very low levels of SOCE as observed in STIM1- or Orai1-deficient T cells sufficient for proliferation (Kim et al., 2011; Ma et al., 2010). Similarly, double knockout of both STIM1 and STIM2, but not individual knockout drastically decreased TCR-induced cell proliferation (Oh-Hora et al., 2008). Collectively, differentiation and cytokine production of effector T cells demand high SOCE, but the threshold levels for T cell proliferation is very low and a minimal increase in $[Ca^{2+}]_i$ is sufficient.

Ca^{2+} signaling in immune suppression

Ca^{2+} signaling in T cells plays a dual role in both positive and negative immune reactions. SOCE in T cells is important for the immune suppressive mechanisms of activated T cell death, anergy, and the function of regulatory T cells. Cell death induced by TCR stimulation is critical for homeostasis of peripheral T cells after antigen clearance (Budd, 2001; Krammer et al., 2007; Strasser, 2005). Therefore, abrogation of T cell death leads to hypersensitive immune reaction and autoimmune disorders. Activation induced T cell death occurs through the death receptor- and mitochondria-mediated pathways. Death receptor-mediated apoptosis involves the Fas ligand (FasL)/Fas signaling pathway, primarily regulated by NFAT (Hodge et al., 1996; Macian et al., 2002; Serfling et al., 2006) while mitochondria-mediated cell death occurs due to loss of mitochondrial membrane potential (Marsden et al., 2003; Strasser, 2005). Mitochondria-mediated cell death pathway involving the Bcl-2 family members (e.g. Bcl-2 and Bcl-X_L) and the BH3-only proteins (e.g. Bad, Bik, Bim, and Noxa) play an important role in T cell survival and death, respectively (Budd, 2001; Hildeman et al., 2007; Hildeman et al., 2002; Marrack et al., 2004; Strasser, 2005; Strasser et al., 2004). It was shown that Orai1-deficient T cells are strongly resistant to cell death due to reduction in both death receptor- and mitochondria-mediated cell death mechanisms by decreasing expression levels of pro-apoptotic genes including FasL and Noxa as well as mitochondrial Ca^{2+} uptake (Kim et al., 2011). Similarly, STIM1 knockout T cells also displayed reduced cell death after mycobacteria infection due to reduced expression of pro-apoptotic factors, which unexpectedly resulted in hyperactive immune response and increased production of IFN-γ in a long term (Desvignes et al., 2015). Collectively, these results suggested that Orai- and STIM-mediated SOCE contributes to

both cell death mechanisms via NFAT-mediated transcriptional regulation and accumulation of Ca^{2+} in the mitochondria.

Orai1 KO T cells show decreased cell death, but heterozygous $Orai1^{+/-}$ T cells display normal level of cell death, suggesting that intermediate levels of $[Ca^{2+}]_i$ are sufficient to induce cell death (Kim KD, and Gwack Y, unpublished). Therefore, the threshold levels of $[Ca^{2+}]_i$ for activated T cell death should be between those of $Orai1^{+/-}$ and $Orai1^{-/-}$ T cells (Fig. 4b). However, as mentioned earlier, $Orai1^{-/-}$ T cells showed a normal rate of proliferation. One possible explanation for the difference in the threshold levels of $[Ca^{2+}]_i$ for proliferation and cell death may be caused by the differential regulation of NFAT1 and NFAT2 transcription factors. Whereas NFAT2 acted as a positive regulator for cell proliferation, NFAT1 induced cell cycle arrest and cell death (Robbs et al., 2008). Another report showed that NFAT1 and NFAT2 are preferentially activated by high and moderate Ca^{2+} levels in T cells, respectively (Srinivasan et al., 2007). Indeed, our RNA-seq analysis of $Orai1^{-/-}$ T cells indicated that expression levels of pro-apoptotic genes, major targets of NFAT1 were drastically decreased while those of positive regulators for cell cycle progress (e.g., c-Myc, CDK4 or CDK6) that are regulated by NFAT2 were not decreased (Mognol et al., 2016) (Srikanth S and Gwack Y, unpublished). Therefore, it is possible that activation of NFAT1, but not NFAT2 is inhibited in $Orai1^{-/-}$ T cells that show decreased cell death with a normal proliferation rate.

Ca^{2+} signaling also plays a pivotal role in the induction of anergy in T cells. Anergic T cells are incapable of proliferation and cytokine expression after antigen encounter (Baine et al., 2009). Technically, it is difficult to apply these standards to determine whether T cells deficient of Orai or STIM proteins undergo anergy because cytokine production in those cells are intrinsically low. However, if we judge only by proliferation after TCR stimulation, Orai1 or STIM1 KO T cells do not seem to undergo anergy because the rates of proliferation of those cells were normal (Kim et al., 2011; Ma et al., 2010). Therefore, the threshold levels of $[Ca^{2+}]_i$ for anergy induction is likely to be higher than $[Ca^{2+}]_i$ levels observed in $Orai1^{-/-}$ or $STIM1^{-/-}$ T cells. Considering the role of Ca^{2+} signaling in inhibition of excessive T cell activation, it seems reasonable that the threshold levels of Ca^{2+} required for negative immune responses including activated T cell death or anergy is higher than that for positive immune reactions.

Dysregulated Ca^{2+} signaling also induces autoimmune and lymphoproliferative disorders by defects in development of regulatory T cells. SCID patients harboring mutations in STIM1 showed autoimmune hemolytic anemia, thrombocytopenia and enlarged spleen and lymph nodes (Picard et al., 2009). Mice lacking both STIM1 and STIM2 showed lymphoproliferative disorder in addition to SCID phenotype (Oh-Hora et al., 2008). The lymphoproliferative phenotype of STIM1/STIM2 double knockout mice was attributed to a severe reduction in thymic Tregs, which are crucial for immune tolerance. These observations identify a strict requirement of the Ca^{2+}-NFAT signaling pathway for the development of Tregs than other T cell types. In addition to T cells, B-cell specific knockout of STIM1 and STIM2 showed decreased expression of the immune suppressive cytokine IL-10, that led to development of autoimmune diseases (Matsumoto et al., 2011). Together, these reports indicated that Ca^{2+} signaling plays an important role in both aspects of immune

regulation - effector T cell activation and immune tolerance, and a block of CRAC channel activity can lead to completely opposite outcomes like immune deficiency or autoimmunity depending on the degree and duration of inhibition.

Future directions

The CRAC channels-calcineurin-NFAT pathway has been extensively studied due to its importance in immune cell functions. Molecular understanding of this fundamental signaling pathway is crucial for development of therapy that benefits patients with immune deficiencies, autoimmune diseases, transplant rejection and cancer. Blockers for calcineurin such as cyclosporin A and FK506 (Tacrolimus) are currently used to suppress transplant rejection and acute inflammation. However, ubiquitous expression of calcineurin makes long-term treatment with the calcineurin blockers technically challenging. Identification of CRAC channel subunits provides potential new targets for drug development. Detailed structural studies targeting the pore region of Orai1 in the closed and open configuration (in the absence and presence of STIM1) will greatly help our understanding of channel regulation. In addition, identification of interacting partners of CRAC channels, particularly those predominantly expressed in the immune system will provide new targets for therapeutic intervention of the Ca^{2+}-calcineurin-NFAT signaling pathway to balance immune reactions. Ca^{2+} is a universal second messenger; however, accumulating evidences suggest that Ca^{2+} signal has specific effects, depending on signaling patterns (e.g., amplitude and frequency of oscillation), site of accumulation (e.g., micro- or nano-domains), and cell types. Unlike other signaling pathways, it is technically possible to fine-tune the strength of Ca^{2+} signaling using genetic manipulation and expression of dominant-negative mutants. Therefore, in vivo studies using these models will provide tools to dissect outcomes of Ca^{2+} signaling under physiological conditions.

The Ca^{2+}-calcineurin-NFAT pathway is a fundamental signaling pathway for T cell physiology including proliferation, differentiation and cytokine production as well as immune suppression. Therefore, molecular dissection of this signaling pathway will definitely provide basic knowledge on various immune phenotypes. However, translational application of inhibitors for CRAC channels need to cross tough barriers derived from different immunological outcomes depending on the levels of Ca^{2+} entry and threshold levels for each phenotype. For example, acute inhibition of CRAC channels can have suppressive therapeutic effects by acutely shutting down effector T cell responses, but in a long term, these effects can be compromised by the lack of immune suppressive mechanisms including T cell death or development of regulatory T cells. Theoretically, we should be able to control the immunological outcomes if we understand the detailed molecular mechanisms that link the intensity and timing of Ca^{2+} signaling to T cell phenotypes.

As mentioned above, Ca^{2+} channels responsible for each step of T cell development have not been identified yet. In addition, many other non-SOC Ca^{2+} channels (e.g., Ca_V, purinergic receptors and transient receptor potential channels) also exist in T cells and play important roles in effector T cell function (Badou et al., 2013) (Nohara et al., 2015). Although deficiency of those channels did not decrease Ca^{2+} entry triggered by TCR stimulation as much as deficiency of Orai or STIM proteins, the immunological outcomes of

inhibition of these channels were significant. These results suggest that their functions are not just supporting CRAC channels, but they may act independently of CRAC channels with the modes of activation, which are different from TCR stimulation (e.g. change in membrane potential or agonists). Therefore, identification of Ca^{2+} channels that function independent of CRAC channels in T cells is another exciting field that needs more attention.

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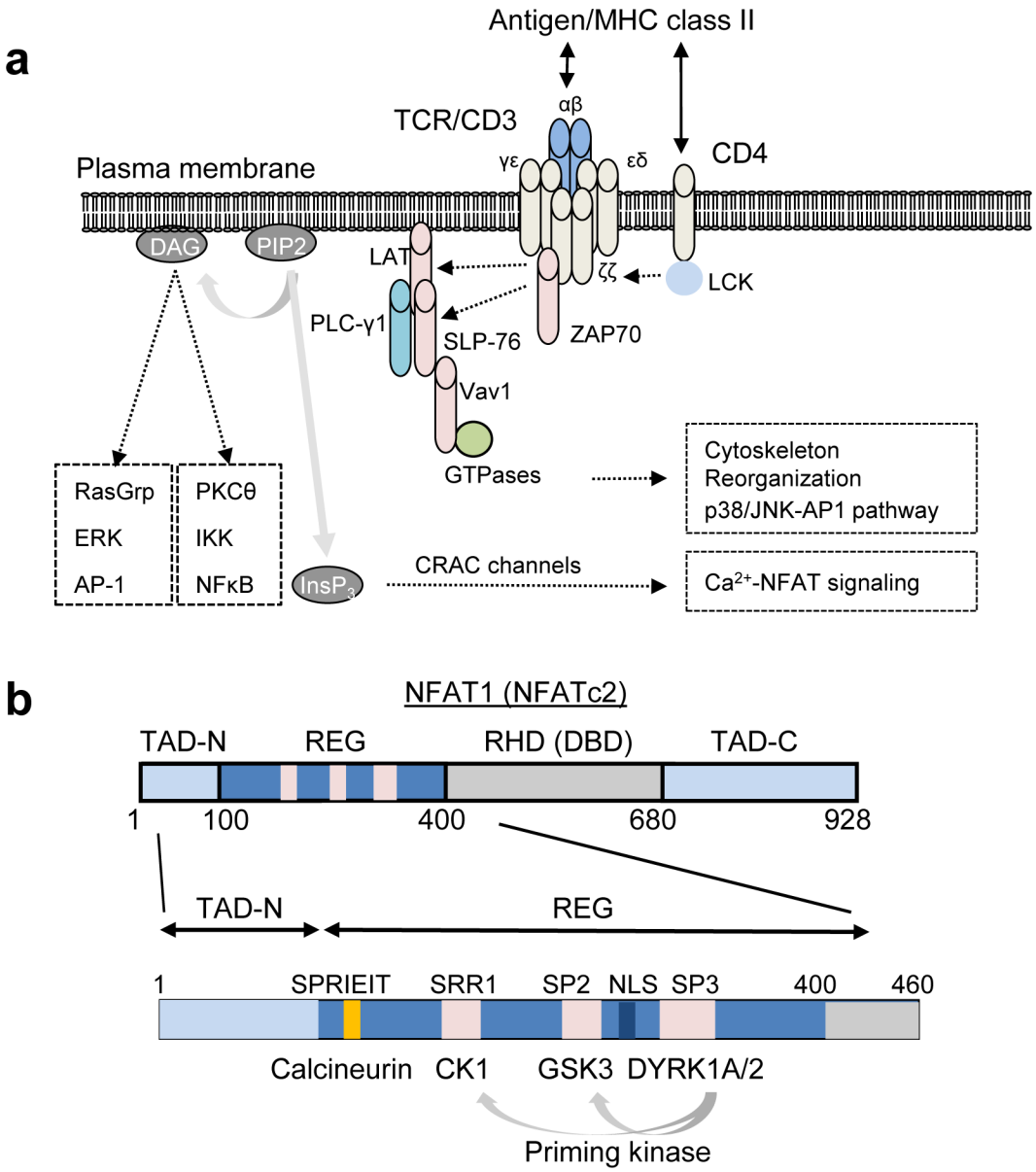

Fig. 1. Signaling pathways of T cell receptor stimulation

(a) Antigen engagement of T cell receptor induces a series of phosphorylation events and activates downstream signaling pathways. Co-receptor (e.g. CD4) ligation to MHC class II positions the protein tyrosine kinase LCK into the antigen-bound TCR/CD3 complex. LCK phosphorylates the cytoplasmic part of ζ chain to recruit ZAP-70. ZAP70 phosphorylates two adaptor proteins LAT and SLP-76 that results in assembly of a signaling complex containing Vav1 and phospholipase C (PLC-γ1). Vav1 recruits downstream effector molecules including Rac1, and a Rho GTPase, CDC42 that have pleiotropic effects in cytoskeleton reorganization and p38/JNK signaling pathways. Vav1 can also recruit large Rab GTPase like CRACR2A-a to activate the JNK signaling pathway. Cytoskeleton reorganization is important for formation of the immunological synapse between antigen presenting cells and T cells. Activated PLC-γ1 hydrolyzes PIP_2 into $InsP_3$ and DAG. While

DAG activates PKCθ–NF-κB and RasGRP-AP1 signaling pathways, $InsP_3$ binds to the $InsP_3$ receptor ($InsP_3R$) on the ER membrane to empty the ER Ca^{2+} store, which induces opening of CRAC channels. Elevated $[Ca^{2+}]_i$ triggers a broad range of downstream signaling pathways including the Ca^{2+}-calmodulin/calcineurin-NFAT pathway.

(b) Schematic of the murine NFAT1 (NFATc2) protein. The transcription activation domains that interact with transcriptional cofactors (e.g. p300) are located at the N and C terminus (TAD-N and TAD-C). DNA binding domain (DBD) shows highest homology with the Rel homology domain (RHD) of Rel-family transcription factors. It also interacts with other transcription factors including AP-1 depending on the DNA sequence. The regulatory domain (REG) contains multiple phosphorylation sites to maintain cytoplasmic localization of NFAT under resting conditions and a docking site for Ca^{2+}-calmodulin-calcineurin complex (SPRIEIT motif). After increase in intracellular $[Ca^{2+}]$, Ca^{2+}-bound calmodulin forms a complex with a protein phosphatase calcineurin, which binds to the SPRIET motif and dephosphorylates the SRR (Serine-rich region) and SP (Ser-Pro-X-X repeat) motifs in the regulatory domain, leading to the nuclear translocation of NFAT1 by exposing the nuclear localization sequence (NLS). SRR1, SP2, and SP3 motifs within the regulatory domain are phosphorylated by casein kinase 1 (CK1), glycogen synthase kinase 3 (GSK3), and dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK) family kinases, respectively. DYRKs play a role as a priming kinase for CK1 and GSK3-mediated phosphorylation.

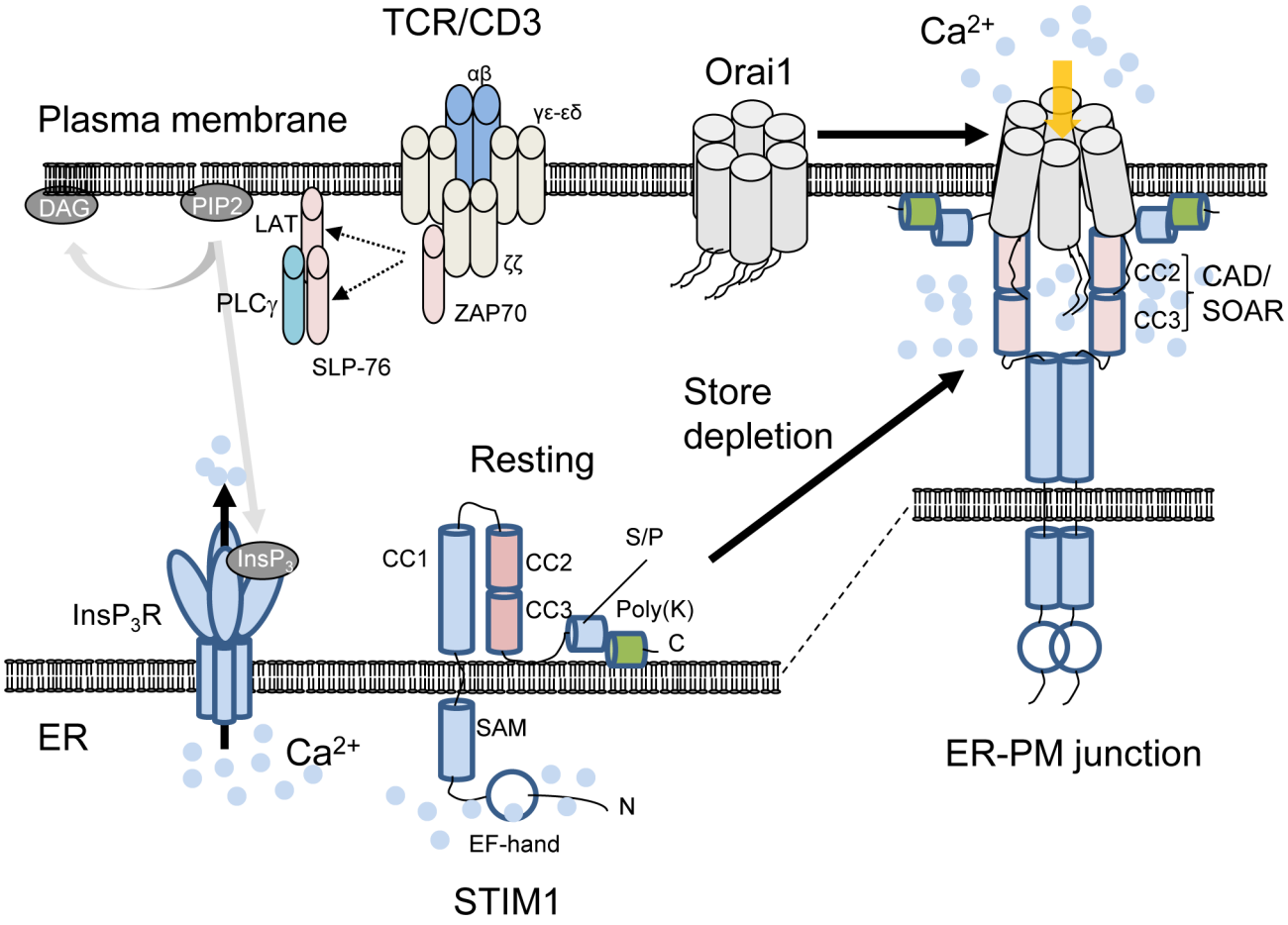

Fig. 2. Activation mechanism of CRAC channels in T cells

Under resting conditions, Orai1 and STIM1 are distributed at the plasma membrane and the ER membrane, respectively. Upon store depletion triggered by T cell receptor stimulation and $InsP_3$ production via PLC-γ1, STIM1 oligomerizes by sensing ER Ca^{2+} depletion with its ER-luminal EF-hand domain and clusters at the ER-plasma membrane junctions. By physical interaction with the cytoplasmic, N and C terminus of Orai1 through the CAD/SOAR domain (coiled coil domains 2 and 3), clustered STIM1 recruits and activates Orai1 at the ER-plasma membrane junctions. STIM1 contains an ER-luminal region comprising the EF-hand and SAM domains, a single transmembrane segment, and a cytoplasmic region. The cytoplasmic region has three coiled-coil domains (CC1, 2, and 3), a serine/proline-rich domain (S/P), and a polybasic tail (poly-K) at the C terminus that interacts with phosphoinositides after store depletion.

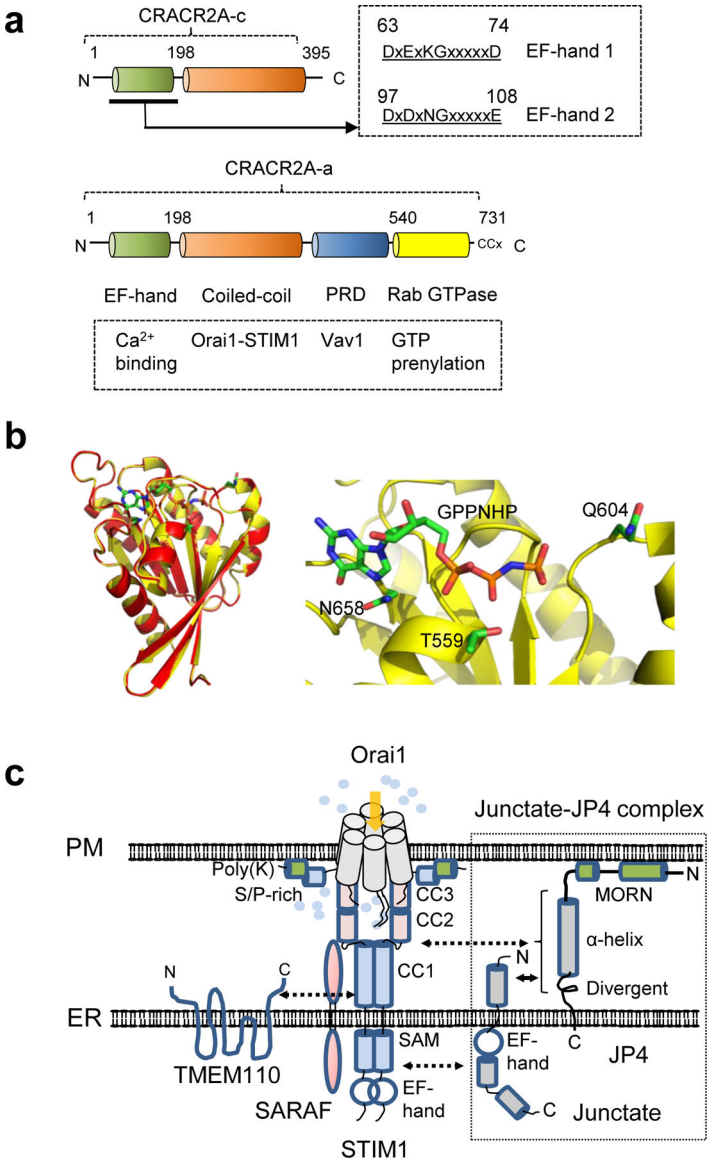

Fig. 3. Interacting partners of Orai1 and STIM1

(a) Schematic showing the domain structure of human CRACR2A-a, a member of the large Rab GTPase family. CRACR2A-a and CRACR2A-c share EF-hand motifs and coiled-coil domain, which interact with and stabilize the Orai1-STIM1 complex. CRACR2A-a contains additional proline-rich domain (PRD), which interact with Vav1 and a Rab GTPase domain with a prenylation site at the C terminus. GTP binding and prenylation are required for the membrane retention of CRACR2A-a.

(b) Homology modeling of CRACR2A-a GTPase domain (yellow) with Rab3a (red). Sequence alignment between GTPase domain of CRACR2A-a and Rab3a gave a continuous alignment with sequence identity of 46% and similarity of 65% (Clustal Omega) (left). MODELLER (Morreale et al., 2000) was used for homology modeling of CRACR2A-a GTPase domain to a high-resolution structure of a GPPNHP-bound Rab3a (PDB ID: 3RAB). A zoomed-in view of the GPPNHP binding site (right). GPPNHP and side-chains

of residues important for GTP binding and hydrolysis, Thr^{559}, Gln^{604} and Asn^{658} are shown in stick representation. A loop consisting of residues 561–570 was removed for clarity. From Srikanth et al (Srikanth et al., 2016a). Reprinted with permission from the American Association for the Advancement of Science (AAAS).

(c) Schematic showing protein interactions among Orai1, STIM1 and auxiliary proteins at the ER-plasma membrane junctions. Under resting conditions, Orai1 and STIM1 are distributed at the plasma membrane and the ER. Upon store depletion, STIM1 oligomerizes by sensing ER Ca^{2+} depletion with its ER-luminal EF-hand domain, and translocates to form clusters at the ER-plasma membrane junctions determined by the junctate-JP4 complex in T cells. By physical interaction with Orai1 through the CAD/SOAR domain (CC2 and CC3, indicated in red), STIM1 recruits and activates Orai1 in the ER-plasma membrane junctions. Junctate also contains Ca^{2+}-sensing motif in the ER lumen and ER Ca^{2+} depletion increases its interaction with STIM1. SARAF and TMEM110 have been shown to translocate together with STIM1 to the ER-PM junctions. SARAF interacts with STIM1 CAD/SOAR domain to modulate slow Ca^{2+} dependent inactivation of CRAC channels while TMEM110 directly interacts with the CC1 region of STIM1 via its cytoplasmic C terminus. TMEM110 is also involved in establishment of ER-plasma membrane junctions.

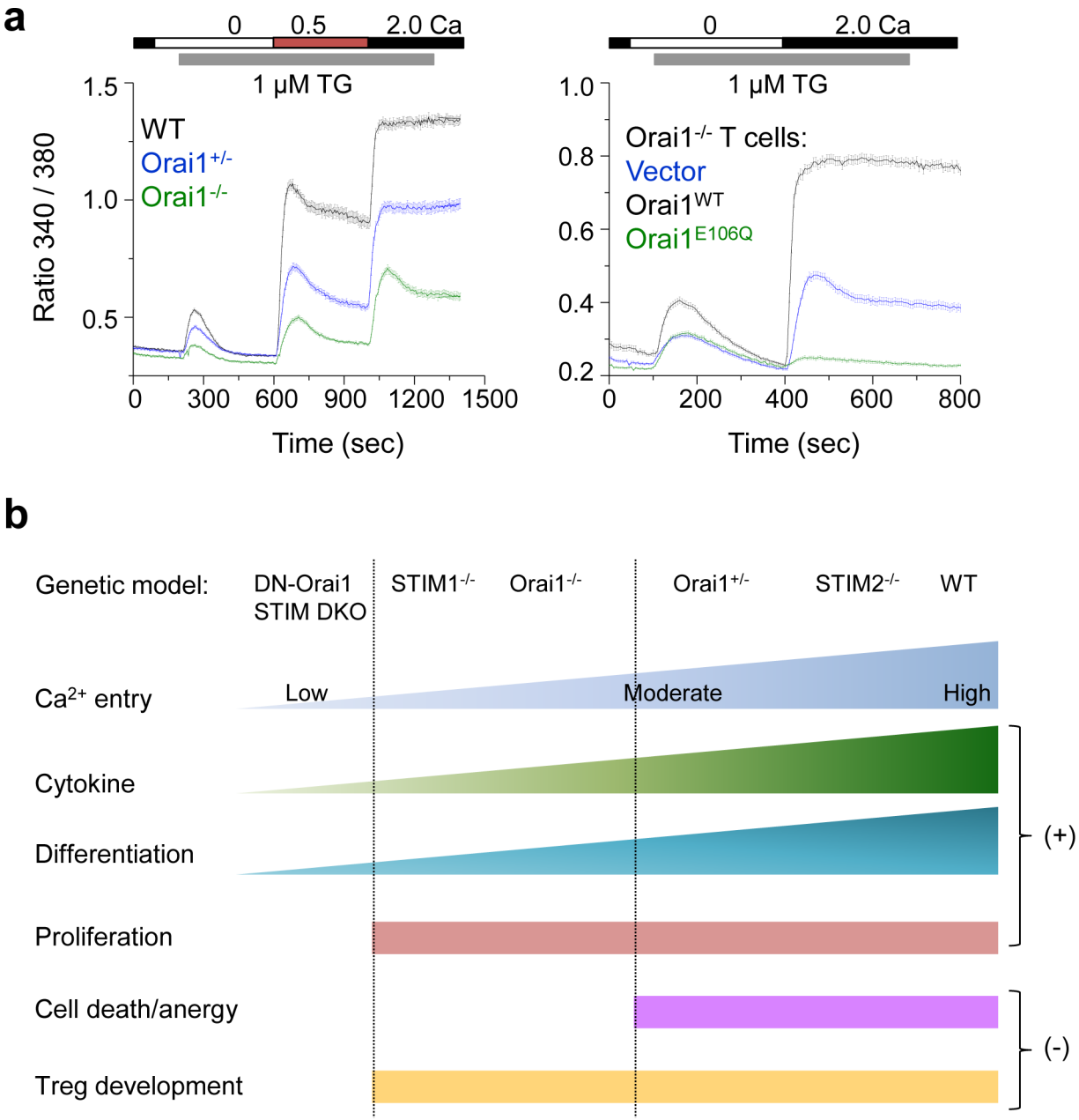

Fig. 4. Role of Ca^{2+} signaling in diverse aspects of T cell activation

(a) Gradual levels of store-operated Ca^{2+} entry generated by genetic modifications. Left - SOCE was measured in effector $CD4^+$ T cells from wild type (WT), Orai1 heterozygous ($Orai1^{+/-}$) and Orai1-deficient ($Orai1^{-/-}$) mice after store depletion with thapsigargin (TG) in the presence of extracellular solution containing 0.5 and 2 mM Ca^{2+}. Right - SOCE was measured in $Orai1^{-/-}$ $CD4^+$ T cells transduced with retroviruses expressing empty vector (vector, blue trace), wild type ($Orai1^{WT}$, black trace) or dominant negative mutant of Orai1 ($Orai1^{E106Q}$). Data modified from article originally published in *The Journal of Immunology* (Kim DK, Srikanth S, Yee MK., Mock DC, Lawson GW, and Gwack Y. 2011. ORAI1

Deficiency impairs activated T cell death and enhances T cell survival. J. Immunol. 187: 3620–3630. Copyright © [2011]. The American Association of Immunologists, Inc.)

(b) Ca^{2+} requirement for cytokine production, differentiation, proliferation, cell death and development of regulatory T cells. Cytokine production and differentiation levels (as determined by expression of signature transcription factors or surface receptors) in effector T cells gradually increases with increasing levels of intracellular Ca^{2+} concentrations. T cell proliferation does not need high Ca^{2+} entry, but requires moderate levels of elevated $[Ca^{2+}]_i$ because $Orai1^{-/-}$ or $STIM1^{-/-}$ T cells showed a normal proliferation rate after stimulation and a further reduction in $[Ca^{2+}]_i$ by overexpression of dominant negative Orai1 (DN-Orai1) in $Orai1^{-/-}$ T cells or deficiency of both STIM1 and STIM2 significantly inhibits proliferation. In some WT T cells, excessive $[Ca^{2+}]_i$ after TCR stimulation induce cell death and anergy, which was decreased by deficiency of Orai1. Therefore, it is likely that inhibition of CRAC channels would result in acute decrease in the immune response by decreasing cytokine production and differentiation of T cells, but in a long term, it can induce autoimmunity and hypersensitive immune responses as outcomes of suppressed cell death, anergy and development of regulatory T cells.