Mol. Cells *35*, 182-194, March 31, 2013
DOI/10.1007/s10059-013-0073-2
eISSN: 0219-1032

Minireview

Molecules and Cells
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Established in 1990

Orai1-NFAT Signalling Pathway Triggered by T Cell Receptor Stimulation

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T cell receptor (TCR) stimulation plays a crucial role in development, homeostasis, proliferation, cell death, cytokine production, and differentiation of T cells. Thus, in depth understanding of TCR signalling is crucial for development of therapy targeting inflammatory diseases, improvement of vaccination efficiency, and cancer therapy utilizing T cell-based strategies. TCR activation turns on various signalling pathways, one of the important one being the Ca^{2+}-calcineurin-nuclear factor of activated T cells (NFAT) signalling pathway. Stimulation of TCRs triggers depletion of intracellular Ca^{2+} store and in turn, initiates store-operated Ca^{2+} entry (SOCE), one of the major mechanisms to raise the intracellular Ca^{2+} concentrations in T cells. Ca^{2+}-release-activated-Ca^{2+} (CRAC) channels are a prototype of store-operated Ca^{2+} (SOC) channels in immune cells that are very well characterized. Recent identification of STIM1 as the endoplasmic reticulum (ER) Ca^{2+} sensor and Orai1 as the pore subunit has dramatically advanced the understanding of CRAC channels and provides a molecular tool to investigate the physiological outcomes of Ca^{2+} signalling during immune responses. In this review, we focus on our current understanding of CRAC channel activation, regulation, and downstream calcineurin-NFAT signaling pathway.

INTRODUCTION

Ca^{2+} is utilized as a second messenger by essentially all cells in unicellular and multicellular organisms, where it regulates diverse aspects of cellular function. Under resting conditions in T cells, cytoplasmic [Ca^{2+}] is in the range of ~100 nM while that in the endoplasmic reticulum, which serves as an intracellular Ca^{2+} store, is much higher (~400 μM). Extracellular [Ca^{2+}] reaches almost 2 mM, establishing a huge [Ca^{2+}] gradient between the extracellular milieu, Ca^{2+} store, and the cytoplasm. Increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) can affect many signaling pathways *via* activation of ubiquitous Ca^{2+} sensors including calmodulin (CaM); which in turn activate a large number of protein kinases/phosphatases and gene transcription, that together shape both the early and late phases of the subsequent cellular response. Ca^{2+} entry *via* store-operated Ca^{2+} (SOC) channels is a predominant mechanism to increase $[Ca^{2+}]_i$ in non-excitable cells, while in excitable cells (e.g. muscle and neuronal cells), voltage-gated ion channels are important for regulation of $[Ca^{2+}]_i$ (Cahalan and Chandy, 2009; Hogan et al., 2010; Lewis, 2011; Putney, 2009). SOC channels were so named because they are activated by depletion of intracellular Ca^{2+} stores (Putney, 1986; 2009). The Ca^{2+}-release-activated-Ca^{2+} (CRAC) channel is a prototype and specialized class of SOC channel in immune cells. Because the volume of ER in T lymphocytes is much smaller than that of other cell types such as cardiac or skeletal muscle cells, SOCE *via* CRAC channels is particularly important to sustain increased $[Ca^{2+}]_i$ necessary for activation of NFAT family of transcription factors. In this review, we will focus on our current understanding of the regulation and known functions of Ca^{2+} signalling in T cells and phenotypes of animal models lacking the components of CRAC channels.

INTEGRATIVE T CELL RECEPTOR SIGNALLING PATHWAYS

Upon pathogen infection, specialized innate immune cells (e.g. dendritic cells) and adaptive immune cells (e.g. B cells) present foreign antigens on their surface together with major histocompatibility complex (MHC) class II molecules. Interactions between TCRs and antigens presented by MHC class II molecules play an important role in T helper cell functions such as differentiation into effector and memory cells, proliferation, and massive cytokine production after full differentiation. 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 and Surh, 2011). Thus, understanding of TCR signalling is crucial for development of therapy to rescue patients with immune deficiencies and to develop pharmacological methods to ameliorate the pathological symptoms of numerous autoimmune diseases exemplified in type I diabetes, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, graft-versus-host disease, and transplant rejection, that are primarily mediated by inflammatory and autoreactive T cells.

Antigen engagement of T cell receptor triggers a cascade of tyrosine phosphorylation events mediated by lymphocyte-

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Received February 28, 2013; accepted March 3, 2013; published online March 11, 2013

Keywords: CRAC channels, NFAT, Orai1, STIM1, T cell receptor

Springer

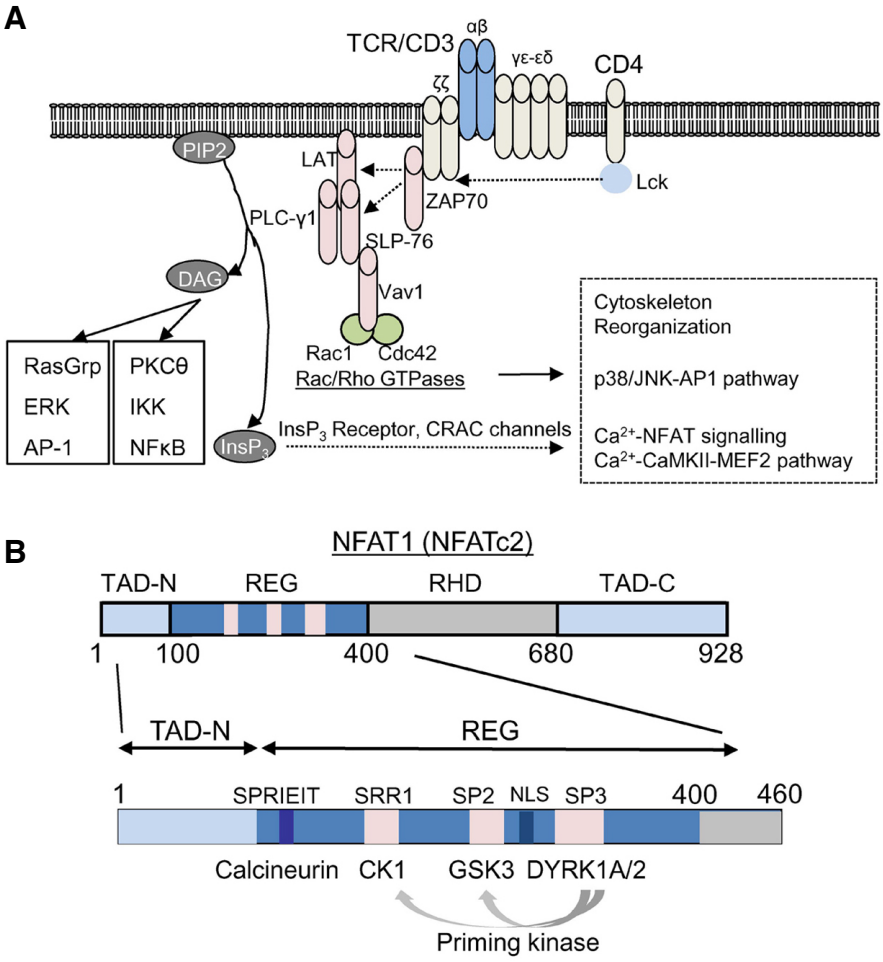

Fig. 1. Signalling pathways of T cell receptor stimulation. (A) Antigen engagement of T cell receptor induces a series of phosphorylation events. Co-receptor (e.g. CD4) ligation in T cells activates protein tyrosine kinase Lck, which phosphorylates the ζ chain of TCR/CD3 complex to recruit ZAP-70 to the TCR/CD3 complex. ZAP70 phosphorylates two adaptor proteins LAT and SLP-76 that results in assembly of a signaling complex containing Vav1 and phospholipase C (PLC-γ1). This signaling complex recruits further downstream effector molecules including Rac1 and a Rho GTPase, cdc42 that have pleiotropic effects in cytoskeleton reorganization, p38/JNK, and Ca^{2+}-NFAT signalling pathways. Cytoskeleton reorganization is important for formation of the immunological synapse between antigen presenting cells and T cells. Activated PLC-γ1 hydrolyzes PIP_2 (phosphatidylinositol 4, 5-bisphosphate) into $InsP_3$ (Inositol 1,4,5 trisphosphate) and DAG (diacyl glycerol). While DAG activates PKC (protein kinase C)-NF-κB and RasGRP1-AP-1 signalling pathways, $InsP_3$ binds to the $InsP_3$ receptor ($InsP_3R$) on the ER membrane to empty the ER Ca^{2+} store. ER Ca^{2+} depletion induces opening of CRAC channels, a prototype of store-operated Ca^{2+} channels. Eleva-ted $[Ca^{2+}]_i$ triggers a broad range of downstream signalling pathways including the Ca^{2+}-calmodulin/calcineurin-NFAT and the Ca^{2+}-CaMKII-MEF2 signaling pathway. Ca^{2+}-bound calmodulin forms a complex with a protein phosphatase calcineurin and dephosphorylates the heavily phosphorylated, cytoplasmic NFAT leading to its nuclear translocation. Nuclear NFAT forms a multi-meric protein complex with itself or with other transcription factors (e.g. AP-1) to induce gene transcription. (B) Schematic of the murine NFAT1 (NFATc2) protein. The transcription activation domains that interact with transcriptional cofactors are located at the N and C terminus (TAD-N and TAD-C). DNA binding domain shows the highest homology with the Rel homology domain of Rel-family transcription factors (RHD). 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 (SPRIET motif). After dephosphorylation by the protein phosphatase complex, the nuclear localization sequence (NLS) within the regulatory domain is exposed leading to nuclear import of NFAT. Serine-rich region (SRR) 1, Ser-Pro-X-X repeat motif (SP) 2, and SP3 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.

specific protein tyrosine kinase (Lck) and zeta chain-associated protein kinase 70 (ZAP70) (Balagopalan et al., 2010; Samelson, 2011; Wang et al., 2010a). These events recruit phospholipase C (PLC) γ1 to the plasma membrane, which hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) into inositol trisphosphate ($InsP_3$) and diacyl glycerol (DAG). DAG predominantly activates NF-κB signalling pathway *via* activation of protein kinase C (PKC) θ, Bcl-10/Carma 1, NF-κB-inducible kinase (NIK), and inhibitor of NF-κB (IκB) kinase (IKK) complex that eventually phosphorylates IκB (Fig. 1A) (Muller and Rao, 2010; Smith-Garvin et al., 2009). Phosphorylation of IκB leads to its degradation and nuclear translocation of NF-κB transcription factors. DAG also activates Ras-mediated signalling pathway *via* activation of Ras guanine nucleotide releasing protein 1 (RasGRP1) which induces phosphorylation-induced activation of AP-1 (Fos-Jun) transcription factors mediated by kinases, dual specificity mitogen-activated protein kinase kinase (MEK) 1/2 and extracellular signal-regulated kinases (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. One of the most studied Ca^{2+}-dependent signaling pathways in the immune system is the Ca^{2+}-calmodulin/calcineurin-NFAT pathway. Upon increase of $[Ca^{2+}]_i$ *via* the CRAC channels, calmodulin binds Ca^{2+} and forms a complex with the protein phosphatase calcineurin, which in turn dephosphorylates the heavily-phosphorylated, cytoplasmic NFAT. Dephosphorylation

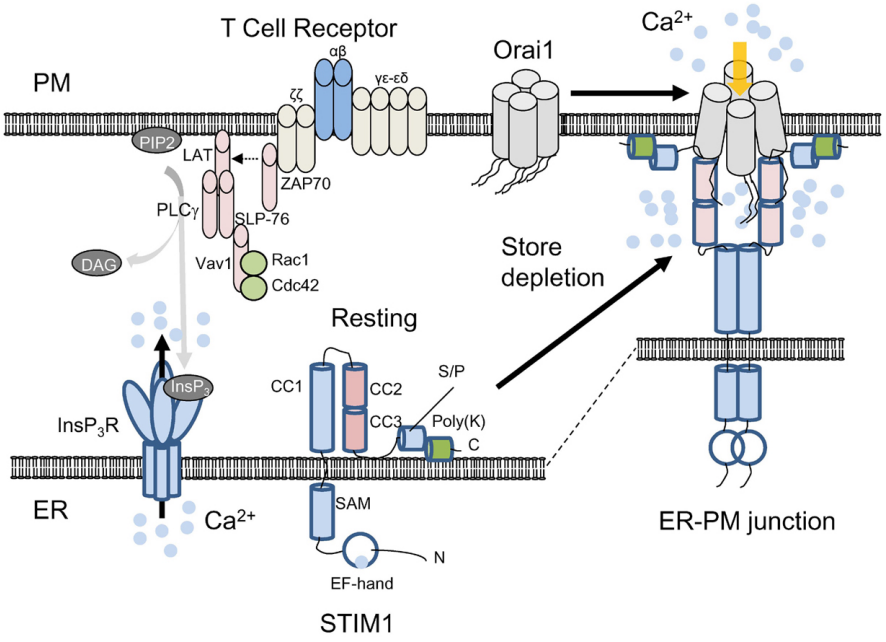

Fig. 2. Activation mechanism of Orai1 and STIM1. Schematic showing current understanding of CRAC channel activation. Under resting conditions, Orai1 and STIM1 are distributed at the PM and the ER membrane. The subunit stoichiometry of Orai1 under resting and stimulated conditions is currently unclear. For convenience, a tetrameric assembly of Orai1 is depicted here. Upon store depletion triggered by T cell receptor stimulation and $InsP_3$ production *via* PLCγ1, STIM1 oligomerizes by sensing ER Ca^{2+} depletion with its ER-luminal EF-hand domain, clusters and translocates to the ER-PM 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 in the ER-PM 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), serine/proline-rich domain (S/P) containing the residues involved in posttranslational modifications (see below), and a polybasic region (poly-K) at the C terminus that interacts with phosphoinositides after store depletion. The exact role of the poly-K tail of STIM1 in resting conditions is not known, but it may interact with phosphoinositides on the ER membrane to maintain its inactive, folded structure.

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 transcription factors (e.g. AP-1) to induce gene transcription involved either in cytokine production, cell proliferation, growth arrest, or cell death, depending on the amplitude and duration of $[Ca^{2+}]_i$ elevation as well as association with other transcription factors (Kim et al., 2011; Macian, 2005; Macian et al., 2002).

In addition to Ca^{2+}-calcineurin-NFAT pathway, increased $[Ca^{2+}]_i$ activates Ca^{2+}-calmodulin-dependent kinase II (CaMKII) that leads to activation of cyclic-AMP-responsive-element-binding protein (CREB) and myocyte enhancer factor 2 (MEF2) (Oh-hora, 2009). Elevated $[Ca^{2+}]_i$ also regulates the Ras signalling pathway by binding to EF-hand motifs of RasGRP1 (Mor and Philips, 2006). The Ras guanine nucleotide exchange factor (RasGEF) activity of RasGRP1 on the Golgi depends on DAG and Ca^{2+} that eventually leads to activation of AP-1 transcription factor. Therefore, Ca^{2+} signalling is integrated with other signalling pathways at the DNA response elements of transcription factors, resulting in cell proliferation, cytokine gene expression, differentiation, or cell death depending on the intensity of diverse signalling pathways.

REGULATION OF NFAT IN T CELLS

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). NFAT consists of four homologous NFAT1 (NFATc2), NFAT2 (NFATc1), NFAT3 (NFATc4), and NFAT4 (NFATc3) (Hogan et al., 2003; Macian, 2005; Serfling et al., 2006; Wu et al., 2007). Most of the NFAT genes are expressed in lymphocytes, however NFAT1 is predominantly expressed in naïve, single-positive T cells. The level of active nuclear NFAT depends both on the intensity of Ca^{2+} influx and on the inducible kinases that are active under a particular stimulation condition. TCR stimulation also induces expression of a short isoform of NFAT2 (NFATc1), NFATc1/αA (Serfling et al., 2012). NFATc1/αA plays a more positive role in T cell activation than the other NFAT family members by supporting proliferation and protecting against cell death upon stimulation.

NFAT proteins contain an N-terminal transactivation domain (TAD), a regulatory domain, a highly conserved DNA-binding domain (Rel-homology domain, RHD) and a C-terminal TAD (Fig. 1B). 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 (Fig. 1B) (Gwack et al., 2007a; Wu et al., 2007). DYRK-family kinases were identified from a genome-wide RNAi screen designed to identify regulators of NFAT localization (Gwack et al., 2006). In a parallel approach, DYRK1A was also identified as an NFAT kinase from the analyses of Down's syndrome critical regions (DSCRs) (Arron et al., 2006). The authors showed that DYRK1A gene lies in the critical region of human chromosome 21 and its high expression caused by trisomy of chromosome 21 accounts for dysregulated NFAT signalling in Down's syndrome. NFAT kinases can be subdivided into two categories based on their function - NFAT export kinases that re-phosphorylate nuclear NFAT for its export into the cytoplasm and NFAT maintenance kinases that phosphorylate cytoplasmic NFAT under resting conditions. CK1 phosphorylates the SRR-1 region of NFAT and

functions as both, maintenance and export kinase. DYRKs phosphorylate the SP-3 motif of NFAT, thereby priming for GSK3- and CK1-mediated phosphorylation of the SP-2 and SRR-1 motifs, respectively. Cytoplasmic DYRK2 serves as the maintenance kinase while nuclear DYRK1A serves as the export kinase. 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 in T cells (Gwack et al., 2007a). 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 (Fig. 1B) (Arron et al., 2006; Gwack et al., 2006). In addition to protein kinases, RNA also plays an important role in the regulation of NFAT localization. Under resting conditions, heavily phosphorylated NFAT proteins exist in a complex with the non-coding RNA Noncoding [RNA] Repressor of NFAT (NRON). 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, exposing its NLS and facilitating nuclear translocation. In the nucleus NFAT can homodimerize or cooperate with multiple transcriptional partners, including AP-1 and forkhead box P-family proteins (FOXP2 and FOXP3) to activate or suppress specific transcriptional programs (Hogan et al., 2003; Wu et al., 2006b).

ESSENTIAL COMPONENTS OF CRAC CHANNELS IN T CELLS, ORAI1 AND STIM1

Although existence of CRAC channels was identified by electrophysiological methods based on their unique biophysical characteristics (Hoth and Penner, 1992), the molecular identity of the channel components was missing. After the completion of genomic sequencing, modern reverse genetics technologies such as genome-wide RNAi screening in *Drosophila* cells implemented by Dr. Norbert Perrimon and colleagues greatly supported identification of the molecular components of CRAC channels (Mohr et al., 2010). Genome-wide RNAi screens in *Drosophila* cells identified the *Drosophila* gene *olf186-F* (named *Drosophila* Orai) and the mammalian homologues Orai1, 2 and 3 as subunits of the CRAC channels (Feske et al., 2006; Gwack et al., 2007b; 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 the patient cells (Feske et al., 2006).

Prior to identification of Orai1, limited RNAi screen in *Drosophila* and HeLa cells identified STIM1, a Ca^{2+}-binding 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). 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 PM-proximal ER, mediates clustering of Orai proteins on the PM, 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 domain 2 and 3 in Fig. 2) was shown to play a pivotal role in activation of Orai1 by direct binding to the cytosolic N and C terminus of Orai1. Furthermore, the stoichiometry of STIM1 binding to Orai1 can affect the fast inactivation properties of CRAC channels, implicating STIM1 as a 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-PM junctions, and gating of Orai1 (Liou et al., 2007; Muik et al., 2008; 2009; Navarro-Borelly et al., 2008; Park et al., 2009; Yuan et al., 2009).

Orai1 has four transmembrane segments (TM1-TM4) with its N and C terminus facing the cytoplasm (Fig. 3A). The TM1 of Orai1 has been shown to line the pore, and residues R91, G98, V102, and E106 in TM1 are important for Ca^{2+} selectivity and gating (Cahalan and Chandy, 2009; Hogan et al., 2010; Lewis, 2011; McNally et al., 2012; Putney, 2009; Zhang et al., 2011). Although TM3 does not line the pore, it was shown that the residues within the TM3 segment including E190 and W176 influence channel gating and ion selectivity indicating a functional relation between TM1 and TM3 (Prakriya et al., 2006; Srikanth et al., 2011). The cytoplasmic N and C terminus of Orai1 mediate channel opening by interaction with STIM1 after store depletion. CRAC channels are also negatively regulated by excess Ca^{2+}, resulting in Ca^{2+}-dependent inactivation (CDI) of the channels (Hoth and Penner, 1992; 1993; Zweifach and Lewis, 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 not only important for channel gating, but also crucial for channel inactivation, to avoid deleterious consequences of excessive Ca^{2+} such as cell death.

PATTERN AND LOCATION OF Ca^{2+} SIGNALS IN IMMUNE CELLS

A general misconception of Ca^{2+} signalling arises from its broad role in downstream events as a universal second messenger. However, many evidences suggest that Ca^{2+} can play a more specialized role in activation of specific signalling pathways depending on the amplitude, oscillation frequency, and the route of 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+} oscillation after TCR engagement that is regulated by a balance between cytoplasmic and ER Ca^{2+} concentrations (Dolmetsch and Lewis, 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 sustained activation of JNK and NF-κB, but not NFAT, whereas prolonged low increases in $[Ca^{2+}]_i$, which were insufficient to activate JNK or NF-κB, sufficed to activate NFAT (Dolmetsch et al.,

1997). Recently, Kar et al. (2012a) showed that different agonists can induce store depletion *via* activating different STIM proteins in rat basophil leukemia (RBL) cells and this process may generate unique Ca^{2+} oscillation patterns. These studies suggest that based on differences in amplitude, duration and stimuli, the same second messenger, Ca^{2+} can activate distinct downstream signalling pathway(s).

Another interesting observation was obtained from comparative studies between Orai1- and transient receptor potential cation (TrpC) 1 channels-mediated Ca^{2+} entry induced by agonist such as carbachol (Ong et al., 2012). Both Orai1 and TrpC1 mediate agonist-induced Ca^{2+} entry; however, only Orai1- mediated Ca^{2+} oscillations efficiently induced nuclear translocation of NFAT, while TrpC1 channel activation led to sustained Ca^{2+} entry at higher agonist concentrations and activated NF-κB-mediated transcription. It was recently shown that $[Ca^{2+}]$ in the microdomains near the CRAC channels are more important for nuclear translocation of NFAT than global increase of Ca^{2+} emphasizing the importance of local Ca^{2+} concentrations in T cells (Kar et al., 2011). These observations are consistent with the results from comparative studies between Ca_V1 and Ca_V2 in activating CaMKII and phosphorylating the cAMP response element-binding protein (CREB) transcription factor, where Ca_V1 has a specialized role in activation of CaMKII by formation of nanodomain(s) of high $[Ca^{2+}]$ (Wheeler et al., 2012). Together, these results indicate that not only the amplitude of Ca^{2+} signalling, but also the pattern (e.g. oscillation frequency, sustained levels, and microdomains) can influence stimulation of diverse downstream signalling pathways.

Upon antigen engagement of $CD4^+$ T cells, Orai1 and STIM1 translocate into the immunological synapse, a site of contact between TCRs and MHC class II molecules with antigen (Barr et al., 2008; Lioudyno et al., 2008). These results indicate that Ca^{2+} entry *via* CRAC channels occurs at specific locations in T cells. The location of Ca^{2+} entry is proven to be the site of Orai1 and STIM1 clustering (Luik et al., 2006). Because mitochondria translocate into the immunological synapse to buffer local Ca^{2+} and inhibit the negative feedback mechanism *via* plasma membrane Ca^{2+} ATPase (PMCA) (Quintana et al., 2011), the specific location of Orai1 and STIM1 clustering can be important for stimulation of specific signaling pathway(s). Ca^{2+} does not play a direct role in the formation of the immunological synapse in a short term because overexpression of the dominant negative mutant of Orai1 did not influence formation of immunological synapse (Lioudyno et al., 2008). However, it may influence the stability of the immunological synapse by suppressing T cell motility indirectly. The physiological role of polarized Ca^{2+} signalling and the molecular mechanism of Orai1 and STIM1 aggregation and retention at the immunological synapse need further investigation. Since the immunological synapse is the site for aggregation of signalling molecules including many tyrosine and serine/threonine kinases, it is possible that CRAC channel clustering and functions are regulated by TCR signalling molecules depending on the signal intensity and costimulation (e.g. CD28 or CTLA-4).

INTERACTING PARTNERS OF ORAI1

After identification of Orai1, numerous regulators and interacting partners of Orai1 have been identified [summarized in Fig. 3A, (Srikanth and Gwack, 2012)]. It will be of interest to determine their role in T cell functions. Proteins interacting with the N terminus of Orai1 include a novel cytoplasmic EF-hand-containing protein, CRAC channel regulator 2A (CRACR2A) (Sri-

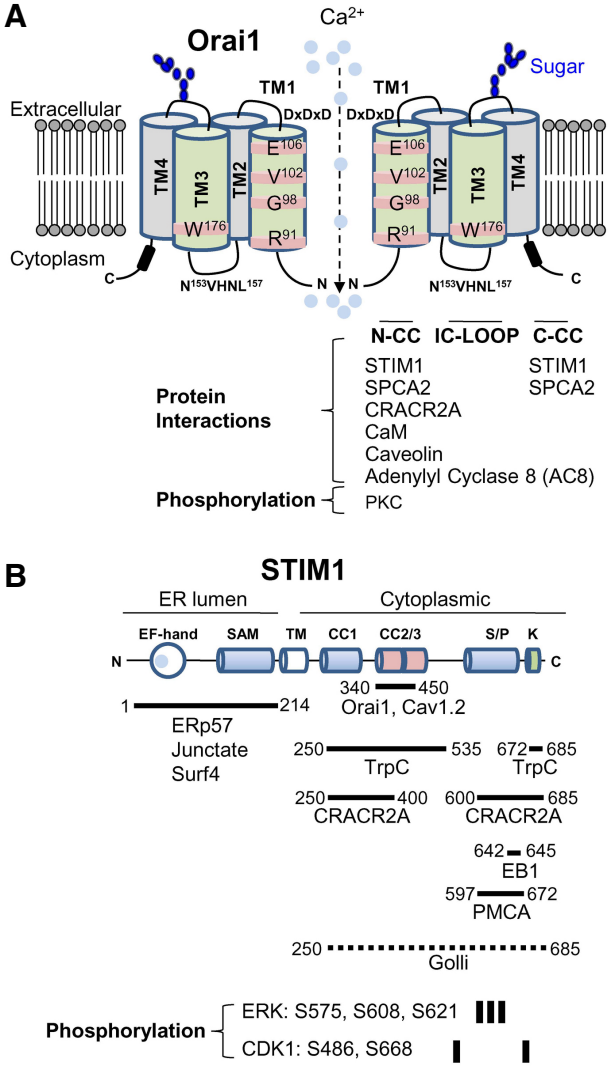

Fig. 3. Interacting partners and posttranslational modification of Orai1 and STIM1. (A) Schematic of Orai1. Orai1 has four transmembrane segments (TM1-TM4). It has two extracellular domains and the second extracellular domain between TM3 and TM4 contains the asparagine (N^{223}) residue involved in glycosylation (indicated in blue). The TM1 lines the pore and the residues in TM1 involved in Ca^{2+} selectivity and gating are depicted. The TM3 does not line the pore, but affects ion selectivity by possible interaction with TM1. Subunits of Orai1 form CRAC channels, but the molecular stoichiometry of CRAC channels is still in question. Schematic depicts a dimeric form for convenience of drawing. Orai1 contains three intracellular domains including the N terminus (N-CC), intracellular loop (IC-LOOP), and C-terminal coiled-coil domain (C-CC) that are important for protein interactions and channel activation/inactivation. Known molecular interactors of Orai1 in these intracellular domains are summarized. The N terminus of Orai1 is phosphorylated by protein kinase C (PKC). (B) Schematic of STIM1. STIM1 contains Ca^{2+}-binding EF hands and a sterile α motif (SAM) domain in the ER-luminal region, a single transmembrane segment, and a long cytoplasmic region. The cytoplasmic region has three coiled-coil domains, serine/proline-rich domain, and a polybasic segment at the C terminus. Proteins associating with each of these domains are indicated. The fragment of STIM1 (340-450) involved in Orai1 interaction/gating is indicated. Golli proteins interact with the cytoplasmic region of STIM1, but the detailed interaction domain is not determined (dotted line). Residues phosphorylated by extracellular signal-regulated kinase (ERK) and cyclin-dependent kinase (CDK1) are indicated.

kanth et al., 2010a). It was shown that CRACR2A forms a ternary complex by direct interaction with Orai1 and STIM1. Interestingly, CRACR2A also regulates Ca^{2+} oscillation in T cells by serving as a cytoplasmic Ca^{2+} sensor. Overexpression of CRACR2A mutant defective in Ca^{2+} binding disrupted Ca^{2+} oscillation pattern (Srikanth et al., 2010a). Recent studies have shown that inhibition of Orai1 and STIM1 activity greatly diminishes cervical and mammary tumor cell migration and metastasis *in vitro* and *in vivo* (Chen et al., 2011; Feng et al., 2010; Yang et al., 2009). Interestingly, an isoform of the secretary pathway Ca^{2+}-ATPase, SPCA2 was shown to enhance mammary tumor cell growth by raising $[Ca^{2+}]_i$ *via* a direct interaction with Orai1 in a STIM-independent manner (Feng et al., 2010).

Calmodulin was identified as a negative regulator of CRAC channels. CaM binds the N terminus of Orai1 at elevated $[Ca^{2+}]_i$ and induces fast Ca^{2+}-dependent inactivation of the channel (Mullins et al., 2009). Another negative regulator of Orai1 function is caveolin. Machaca and colleagues used xenopus oocyte as a model and identified a role for caveolin in internalization of Orai1 (Yu et al., 2010). It was shown that during meiosis, SOCE is inhibited due to internalization of Orai1 and impairment of STIM1 clustering (Yu et al., 2009). The authors showed that Orai1 internalization occurred *via* a caveolin and dynamin-dependent endocytic pathway and mapped a caveolin-binding site in the N terminus of Orai1 and demonstrated protein interaction between Orai1 and caveolin (Yu et al., 2010).

Recently, a crosstalk between the CRAC channel pathway and cyclic adenosine monophosphate (cAMP) signaling pathway has been identified. An important intracellular signaling molecule cAMP is generated by adenylyl cyclase (AC)-mediated cleavage of adenosine triphosphate, which in turn activates downstream PKA pathway. Among the nine membrane-bound ACs, four of them (AC1, AC8, AC5 and AC6) are known to be regulated by Ca^{2+}. Of those four, AC1 and AC8 are stimulated through an interaction with Ca^{2+}-CaM complex. A role of STIM1 in activation of AC activity has been demonstrated (Lefkimmiatis et al., 2009). It was shown that intracellular store depletion, independent of cytosolic Ca^{2+} concentration, led to recruitment of ACs in a STIM1-dependent mechanism referred by the authors as “store-operated cAMP signalling”. Another study demonstrated a direct protein interaction between Ca^{2+}-CaM activated AC8 and Orai1 N terminus (Willoughby et al., 2012). Using Forster resonance energy transfer (FRET) technique, GST pulldown, and immunoprecipitation analyses, the authors detected a constitutive association between Orai1 and AC8, which was not affected by store depletion and Orai1 activation (Willoughby et al., 2012). Both these studies together show that STIM1 itself or Ca^{2+} entry *via* Orai1 plays an integral role in regulating crosstalk between SOCE and cAMP signaling pathways.

BINDING PARTNERS OF STIM1

Only a few positive regulators of STIM1 function have been identified so far (Fig. 3B). Previous results showed that Orai1 and STIM1 translocate into pre-existing junctional areas, a space of 10-25 nm between the PM and ER membranes (Varnai et al., 2007; Wu et al., 2006a). STIM1 contains a polybasic stretch of amino acids in its C terminus that bind phosphoinositides, and is important for its clustering at ER-PM junctions (Korzeniowski et al., 2009; Liou et al., 2007; Walsh et al., 2010). Truncation of this polybasic domain abolished STIM1 accumulation at ER-PM junctions, and overexpression of Orai1 recovered accumulation of STIM1-ΔK mutant into the ER-PM junctions (Liou et al., 2007; Park et al., 2009). In excitable cells (e.g. neurons and muscle cells), proteins including junctophiins, junctin, and junctate localize to the junctions between the PM and ER/sarcoplasmic reticulum (SR) membranes and form a structural foundation for regulating the intracellular Ca^{2+} stores and Ca^{2+} entry (Berridge et al., 2003; Carrasco and Meyer, 2011; Takeshima et al., 2000; Weisleder et al., 2008). It was shown that that junctate is a structural component of the ER-PM junctions in T cells and recruits STIM1 into these junctions after store depletion suggesting a conserved function of the components of the ER-PM junctions in excitable and non-excitable cells (Srikanth et al., 2012).

Numerous negative regulators of STIM1 have been identified. An ER resident protein SARAF (SOCE-associated regulatory factor; TMEM66) was identified as an interacting partner of STIM1 that facilitates the Ca^{2+}-dependent slow inactivation of CRAC channels (Palty et al., 2012). SARAF was shown to interact with STIM1 *via* its cytoplasmic C terminus containing a serine-proline rich domain and polybasic region under resting conditions. After store depletion, SARAF was shown to translocate to the ER-PM junctions with STIM1 and facilitate the slow inactivation of CRAC channels. Surfeit locus protein 4 (Surf4) was also identified as an interacting partner of STIM1 (Fujii et al., 2012). Surf4 interacted with the ER-luminal region of STIM1 and its deficiency increased STIM1 translocation, thus SOCE. A surface plasmon resonance screen monitoring the changes in resonance signal between immobilized ER-luminal N terminus of STIM1 on a chip and various ER resident proteins identified a 58-kDa thiol oxidoreductase ERp57 as an ER-intraluminal binding partner and a negative regulator of STIM1 (Prins et al., 2011). Polycystin-1 fragment P100 and Stanniocalcin 2 (STC2) have been shown to interact with STIM1 and negatively regulate its translocation. Mutations in polycystin-1 (PC-1) or polycystin-2 (PC-2) encoding genes result in autosomal dominant polycystic kidney disease (ADPKD). PC-1 cleavage fragment of 100-kDa, P100, inhibited SOC currents when expressed in *Xenopus* oocytes (Woodward et al., 2010). Another study identified stanniocalcin 2, a homologue of stanniocalcin 1, a secreted glycoprotein involved in Ca^{2+} uptake during hypercalcemia, as a negative regulator of SOCE (Zeiger et al., 2011). Stanniocalcin 2 was shown to interact with STIM1, however, the molecular mechanism of how it inhibited SOCE remains unknown. STIM1 was identified as binding partner of EB1, a microtubule tip-binding protein, and shown to play a role in ER tubulation (Grigoriev et al., 2008). Recently, a novel protein of unknown function, POST (partner of STIM1, TMEM20) was also identified as a STIM1 interactor by affinity purification of Orai1 from Jurkat T cells (Krapivinsky et al., 2011). POST is a 10-transmembrane containing protein localized to the PM and ER membrane. While POST expression did not affect STIM1-Orai1 induced CRAC currents, the authors suggested a role of POST in regulating plasma membrane Ca^{2+} ATPase (PMCA) activity.

STIM1 is also known to interact with and regulate ion channels in addition to Orai1. An interaction between STIM1 and TrpC channels has been studied [(Huang et al., 2006; Ong et al., 2007; Worley et al., 2007), but also see (DeHaven et al., 2009)]. The arachidonic acid-regulated Ca^{2+} (ARC) channels, a class of highly Ca^{2+}-selective ion channels, activated by low concentrations of arachidonic acid are opened by the pool of STIM1 that constitutively resides in the PM (Mignen et al., 2007). In addition to the ARC channels, two groups showed an interaction between STIM proteins and $Ca_V1.2$ channels (Park et al., 2010; Wang et al., 2010b). The CAD/SOAR fragment of

STIM1 required for Orai1 activation was shown to inactivate and inhibit surface expression of $Ca_V1.2$ (Fig. 3B) (Park et al., 2010; Wang et al., 2010b).

MULTIPLE Ca^{2+} SENSORS REGULATE CRAC CHANNEL ACTIVITY

The protein interaction studies suggest an interesting scenario where multiple Ca^{2+}-sensing molecules are involved in regulation of CRAC channel activity. Under resting conditions, Orai1 and STIM1 are distributed at the PM and the ER membrane, respectively. Ca^{2+}-bound STIM1 exists as a folded structure mediated by intramolecular interaction between the CAD/SOAR domain and the autoinhibitory region within the coiled-coil domain (Korzeniowski et al., 2010). Junctate also exists as a Ca^{2+}-bound form *via* its Ca^{2+} binding domains in the ER lumen (Fig. 4A). Cytoplasmic Ca^{2+} sensors such as CaM and CRACR2A do not bind Ca^{2+} under resting conditions. Upon store depletion, STIM1 loses bound Ca^{2+}, unfolds itself, oligomerizes, and translocates to form clusters at the ER-PM junctions premarked by junctate protein (Fig. 4B). ER store depletion causes junctate to lose bound Ca^{2+} and trap STIM1 into the ER-PM junctions by direct interaction. At this stage, cytoplasmic CRACR2A stabilizes Orai1-STIM1 complex by protein interaction. As $[Ca^{2+}]_i$ increases, CRACR2A binds Ca^{2+} *via* its EF-hands and dissociates from the Orai1-STIM1 complex and Ca^{2+}-bound CaM is recruited into the Orai1-STIM1 complex to inactivate the CRAC channels (Fig. 4C). When ER Ca^{2+} is re-filled by SERCA following the increase of $[Ca^{2+}]_i$, the protein complex of Orai1 and STIM1 dissociates and returns to the resting state.

POSTTRANSLATIONAL MODIFICATION OF ORAI1 AND STIM1

Posttranslational modifications have been shown to regulate SOCE. Kawasaki et al showed that Orai1 was phosphorylated at S27 and S30 in the cytoplasmic N terminus by protein kinase C (PKC) β and this phosphorylation suppressed SOCE (Kawasaki et al., 2010) (Fig. 3A). Cells expressing Orai1 mutated at S27 and S30 showed a marginal enhancement of SOCE and CRAC currents (Kawasaki et al., 2010). Interestingly, Orai proteins contain several conserved serine residues, some in the N terminus close to the pore-forming TM1 (S75, S82, S89, and S90; amino acid numbering based on human Orai1), in the intracellular loop (S159 and S163), and C terminus (S260). Based on their location, it is possible that phosphorylation of these residues may influence the regulation of channel gating, inactivation, or protein interactions of Orai1. Future studies examining the mutations of these residues and their impact on SOCE and CRAC currents would be important to understand phosphorylation-mediated regulation of Orai proteins.

Putney and colleagues demonstrated phosphorylation mediated regulation of STIM1 and SOCE during mitosis (Smyth et al., 2009) (Fig. 3B). It was known that SOCE was suppressed during cell division, however, the molecular mechanism behind this event was not known. Smyth et al showed that S486 and S668 located within the serine/threonine-rich domain of STIM1 were phosphorylated during mitosis by cyclin dependent kinase 1 (CDK1) and these phosphorylations suppressed SOCE. Expression of STIM molecules that were truncated at position 482 or with mutations of S486 and S668 significantly rescued SOCE in mitotic cells (Smyth et al., 2009). In a different study, Machaca and colleagues showed that SOCE was suppressed even in meiotic cells. The authors observed trapping of Orai1 in

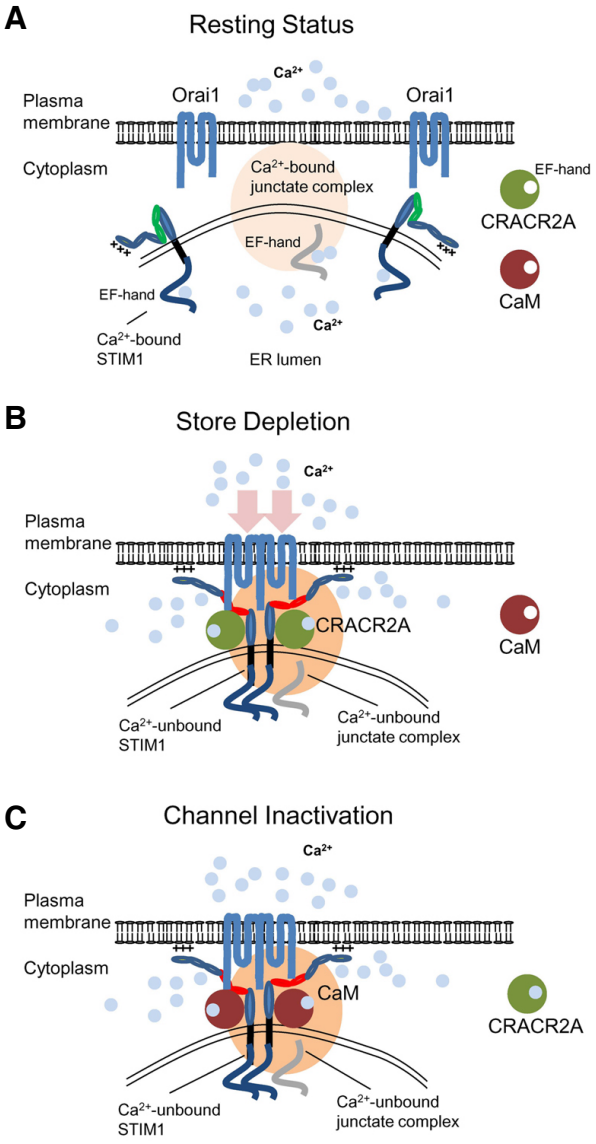

Fig. 4. CRAC channel regulation by multiple Ca^{2+}-sensing molecules in the ER and cytoplasm. (A) Schematic showing a possible mechanism of CRAC channel regulation. Under resting conditions, Orai1 and STIM1 are distributed at the PM and the ER membrane. Junctate is located at ER-PM junctions in a Ca^{2+}-bound form *via* its ER-luminal EF hand domain (indicated in gray). Cytoplasmic Ca^{2+} sensors such as CRACR2A and calmodulin are not bound to Ca^{2+} in resting conditions. (B) 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-PM junctions. By physical interactions with Orai1 through the CAD/SOAR domain (depicted in red), clustered STIM1 recruits and activates Orai1 in the ER-PM junctions. During the process, junctate loses bound Ca^{2+} and supports STIM1 recruitment into ER-PM junctions. CRACR2A is recruited into the Orai1-STIM1 complex to stabilize their interactions. (C) Following the increase of cytoplasmic $[Ca^{2+}]$, CRACR2A dissociates from the Orai1-STIM1 complex. Ca^{2+}-bound calmodulin interacts with the N terminus of Orai1 and inactivates the channel *via* a mechanism called fast inactivation. The slow inactivation of CRAC channels depends on Ca^{2+} entry and interaction with SARAF. After channel inactivation, once the ER is refilled with Ca^{2+}, Orai1 and STIM1 return to the resting status.

intracellular vesicles and impaired STIM1 clustering in xenopus oocytes during meiosis (Yu et al., 2009). Overexpression of $STIM1^{D76A}$, a constitutive active mutant also showed impaired cluster formation, suggesting that STIM1 multimerization was blocked during meiosis. A study by Pozo-Guisado et al. (2010) identified a positive role of phosphorylation of STIM1 on SOCE. The authors showed that ERK1 and ERK2 phosphorylate S575, S608, and S621 residues located within the C-terminal S/T-rich domains of STIM1 (Fig. 3B) (Pozo-Guisado et al., 2010). These phosphorylation events were induced by store depletion with thapsigargin as well as ERK agonist, 12-O-tetradecanoylphorbol-13-acetate (TPA). FRET experiments measuring interaction between Orai1 and STIM1 showed that phosphorylation enhanced STIM1 association with Oria1 when compared with a mutant STIM1 that cannot be phosphorylated by ERK1/2 (Pozo-Guisado et al., 2010). Another study reported a positive role of tyrosine phosphorylation of STIM1 in human platelets (Lopez et al., 2012). STIM1 was shown to be phosphorylated at tyrosine residues by immuno-blotting with a classical anti-phospho-tyrosine antibody, 4G10. This event is possibly mediated by Bruton's tyrosine kinase (Btk) because a specific blocker of Btk, LFM-A13 suppressed tyrosine phosphorylation of STIM1 and protein interactions between Orai1 and STIM1 (Lopez et al., 2012).

DIVERSE ROLES OF CRAC CHANNEL-MEDIATED Ca^{2+} SIGNALLING IN IMMUNE CELLS

Several reports have described the phenotypes of Orai1- and STIM1-deficient immune cells (Bergmeier et al., 2009; Braun et al., 2009a; Gwack et al., 2008; Oh-Hora et al., 2008; Schuhmann et al., 2010; Vig et al., 2008). In humans, loss of Orai1 and/ or STIM1 function causes immune deficiency and symptoms of these patients have been recapitulated in animal models lacking expression of Orai1 or STIM1 proteins. ORAI1-deficient mice showed a reduction in cytokine production by $CD4^+$, $CD8^+$ effector T cells and mast cells (Gwack et al., 2008; Vig et al., 2008). In addition, ORAI1 deficiency impaired Ca^{2+} influx and functions of neutrophils and platelets (Bergmeier et al., 2009; Braun et al., 2009b; Schaff et al., 2010). STIM1 deficiency 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., 2010). 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., 2010). These results clearly demonstrate a predominant role of Orai1 and STIM1 in effector T cell functions *in vivo*.

In addition to a positive role in immune activation, Ca^{2+} signalling also plays a role in negative regulation of the immune system [summarized in (Qu et al., 2011)]. Dysregulated Ca^{2+} signalling also induces autoimmune and lymphoproliferative disorders. SCID patients harboring mutations in STIM1 also 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 double knockout mice was attributed to a severe reduction in regulatory T cells (Tregs) which are crucial for immune tolerance (Oh-Hora et al., 2008). These observations identify an important role for Ca^{2+}-NFAT signaling pathway in development of Tregs (Wu et al., 2006b). In addition, B-cell specific knockout of STIM1 and STIM2 showed decreased levels 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+} signalling 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 and autoimmunity.

Another role of Ca^{2+} signalling in immune suppression is observed when T cells undergo cell death. Cell death induced by TCR stimulation is critical for homeostasis of peripheral T cells after antigen clearance and for negative selection of auto reactive T cells in the thymus (Budd, 2001; Krammer et al., 2007; Strasser, 2005). 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/Fas signalling 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 and Strasser, 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 death and survival as seen in isolated T cells and in animal models (Budd, 2001; Hildeman et al., 2002; 2007; Marrack and Kappler, 2004; Strasser, 2005; Strasser and Pellegrini, 2004). It was shown that Orai1-deficient T cells are strongly resistant to cell death due to reduction in death receptor- and mitochondria-mediated cell death mechanisms by decreasing expression levels of proapoptotic genes and mitochondrial Ca^{2+} uptake (Kim et al., 2011). These results suggested that Ca^{2+} signalling contributes to both cell death mechanisms *via* accumulation of Ca^{2+} in the mitochondria and NFAT-mediated transcriptional regulation. A positive role of Orai1 in cell death is also observed in various cell types with different inducers of cell death such as soft substrate, oxidation, and ER stress (Chiu et al., 2008; Flourakis et al., 2010; Hawkins et al., 2010; Henke et al., 2013). However, its negative role in cell death with activation of CD95 (Fas) and type I interferon has also been demonstrated (Khadra et al., 2011; Yue et al., 2012). Therefore, it seems that the role of Ca^{2+} entry *via* Orai1 in cell death cannot be generalized; instead, different ligands and inducers may have unique Ca^{2+} patterns as discussed above that can influence the outcome of cell death or survival.

The role of CRAC channels in T cell proliferation seems to be more complex than in cytokine production or cell death because it differs significantly in various mouse models of CRAC channels. It was shown that $Orai1^{-/-}$ T cells proliferate normally although they show a severe reduction in cell death triggered by TCR stimulation (Kim et al., 2011). These results suggest that different thresholds of $[Ca^{2+}]_i$ may be necessary for different physiological outcomes, with proliferation requiring much lesser $[Ca^{2+}]_i$ than cell death. A further reduction in $[Ca^{2+}]_i$ by expression of a dominant negative mutant of Orai1 in $Orai1^{-/-}$ T cells almost completely abolished TCR-induced cell proliferation (Fig. 5A). It was shown that other Orai family members, Orai2 and Orai3, possibly contribute the residual Ca^{2+} entry in $Orai1^{-/-}$ T cells. Hence the dominant negative mutant $Orai1^{E106Q}$ is likely to heteromultimerize with Orai2 and Orai3 in $Orai1^{-/-}$ T cells to further decrease SOCE (Kim et al., 2011). Orai1 deficiency shows a gene dosage effect in both human and mouse T cells with heterozygotes showing intermediate levels of Ca^{2+} entry (Feske et al., 2006; Gwack et al., 2008; Kim et al., 2011). Inter-

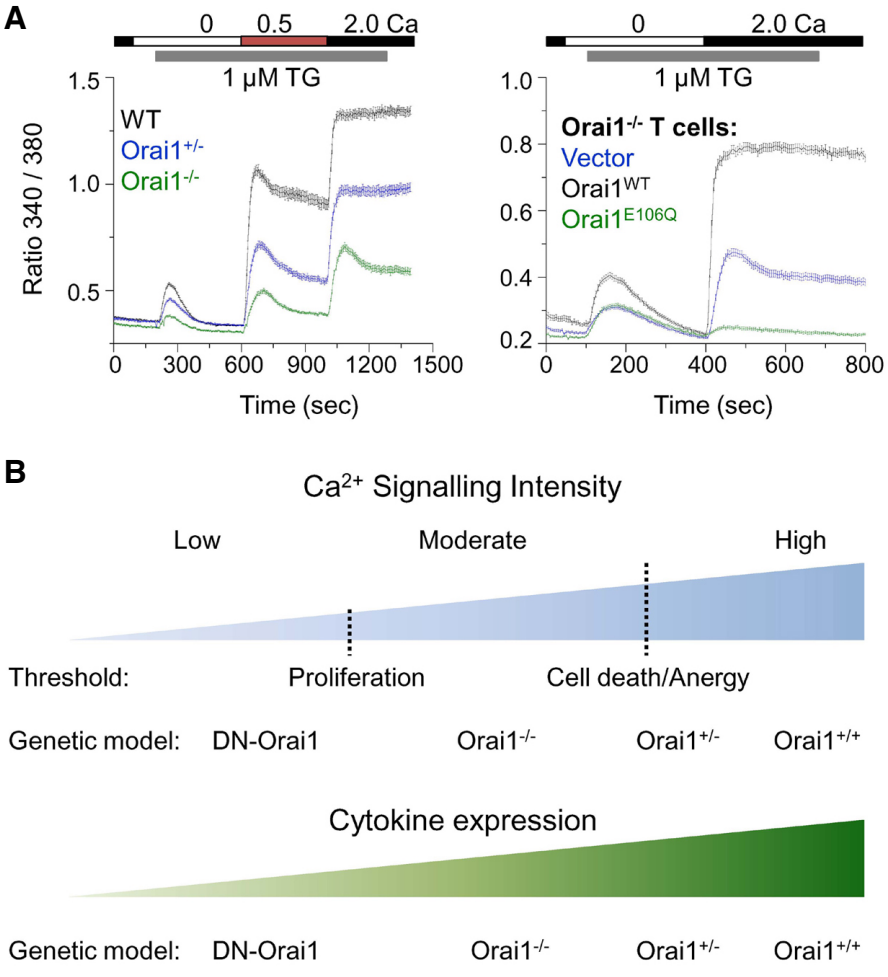

Fig. 5. Roles of Ca^{2+} signalling in diverse aspects of T cell activation. (A) Gradual levels of store-operated Ca^{2+} entry generated by genetic modifications. Left - CRAC channel activity was measured in effector $CD4^+$ T cells from wild type (WT), Orai1 heterozygous ($Orai1^{+/-}$) and Orai1-deficient ($Orai1^{-/-}$) mice after store depletion with thapsigargin (TG) in the presence of extracellular solution containing 0.5 and 2 mM Ca^{2+}. Right - CRAC channel activity 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 (Kim et al., 2011). (B) Ca^{2+} requirement for T cell death, cytokine production and proliferation differs. T cell proliferation does not need high Ca^{2+}. Instead, excessive Ca^{2+} concentrations induce cell death and anergy in T cells. Therefore, T cell proliferation requires moderate intracellular Ca^{2+} concentrations as observed in Orai1-deficient ($Orai1^{-/-}$) T cells and a further reduction in $[Ca^{2+}]_i$ by overexpression of dominant negative Orai1 (DN-Orai1) in $Orai1^{-/-}$ T cells inhibits proliferation. Thus the threshold of $[Ca^{2+}]_i$ necessary for proliferation is much lower than that for cell death and anergy. However, cytokine production gradually increases with increase in $[Ca^{2+}]_i$, with DN-Orai1 cells showing minimal cytokine production and $Orai1^{+/+}$ cells showing maximal cytokine levels. Thus, the pattern of Ca^{2+} signalling can modulate the outcomes of T cell fates such as cytokine production, proliferation, anergy, and cell death in a digital or analogue manner.

estingly, $Orai1^{+/-}$ mice do not show any SCID phenotype or defect in cell death suggesting that intermediate levels of $[Ca^{2+}]_i$ are sufficient to induce cell death. 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). Repetitive TCR stimulation is unlikely to induce anergy in $Orai1^{-/-}$ T cells because proliferation and cell cycle progression were actively induced. Therefore, the threshold levels of $[Ca^{2+}]_i$ for anergy induction is likely to be higher than that observed in $Orai1^{-/-}$ T cells. These studies delineate the thresholds of $[Ca^{2+}]_i$ with extremely low $[Ca^{2+}]_i$ (as seen in $Orai1^{-/-}$ T cells expressing dominant negative $Orai1^{E106Q}$) blocking both proliferation and cell death, slightly higher levels (as observed in $Orai1^{-/-}$ T cells) sufficient for proliferation but not for cell death, and intermediate levels (as observed in $Orai1^{+/-}$ T cells) sufficient for both proliferation and cell death (Fig. 5B). These observations are similar with the concept proposed by Parekh and colleagues indicating that graded Ca^{2+} influx *via* CRAC channels (analog signal) induces all-or-none activation of gene transcription *via* NFAT (digital outcome) (Kar et al., 2012b). However, all the outcomes of graded Ca^{2+} influx are not digital, because T cells with different levels of $[Ca^{2+}]_i$ showed a gradual effect in cytokine production with increasingly higher cytokine levels produced by $Orai1^{-/-}$ $CD4^+$ T cells expressing $Orai1^{E106Q}$, $Orai1^{-/-}$, $Orai1^{+/-}$, and $Orai1^{+/+}$ T cells in an analogous manner. Further studies using transgenic animal models exhibiting graded Ca^{2+} influx will help in dissecting the digital and analog outcomes of Ca^{2+} signaling under physiological conditions.

CONCLUSION

Molecular understanding of CRAC channels is crucial for development of therapy that benefits patients with immune deficiencies, autoimmune diseases, and transplant rejection. Ca^{2+} signalling pathway comprising of CRAC channels-calcineurin-NFAT has been extensively studied due to its importance in immune cell functions. Blockers for calcineurin such as cyclosporin A and FK506 (Tacrolimus) are currently used as strong immunosuppressants 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 and understanding of their regulation now provide new targets

for drug development to prevent transplant rejection and treat autoimmune diseases. Blockers of CRAC channels are less likely to have detrimental side effects because SCID patients with defective CRAC channel activity showed mild extra-immunological phenotype. 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 because this will provide useful information for designing of chemicals specifically targeting Orai1 pore. In addition, identification of interacting partners of CRAC channels, particularly in the immune system will provide new targets for therapeutic intervention of the Ca^{2+}-calcineurin-NFAT signaling pathway. Ca^{2+} is a universal second messenger; however, accumulating evidences suggest that $[Ca^{2+}]_i$ has specific effects depending on signaling patterns (e.g. amplitude and frequency of oscillation), site of accumulation (e.g. micro- or nanodomains), and cell types. *In vivo* studies using transgenic animal models exhibiting graded Ca^{2+} influx will provide tools to dissect the digital and analog outcomes of Ca^{2+} signaling under physiological conditions. In addition to the immune system, recent identification of the role of Orai1 in cervical and mammary tumor cell migration and metastases provides new opportunities for targeting Orai1 as cancer therapy and for investigating the function of intracellular $[Ca^{2+}]_i$ in tumor formation.

ACKNOWLEDGMENTS

This work was supported by National Institute of Health grants AI-083432 and AI-101569, a grant from the Lupus Research Institute (Y.G.), and a scientist development grant from the American Heart Association, 12SDG12040188 (S.S.).

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