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Calcium signalling in T cells

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Abstract

Calcium (Ca^{2+}) signalling is of paramount importance to immunity. Regulated increases in cytosolic and organellar Ca^{2+} concentrations in lymphocytes control complex and crucial effector functions such as metabolism, proliferation, differentiation, antibody and cytokine secretion and cytotoxicity. Altered Ca^{2+} regulation in lymphocytes leads to various autoimmune, inflammatory and immunodeficiency syndromes. Several types of plasma membrane and organellar Ca^{2+}-permeable channels are functional in T cells. They contribute highly localized spatial and temporal Ca^{2+} microdomains that are required for achieving functional specificity. While the mechanistic details of these Ca^{2+} microdomains are only beginning to emerge, it is evident that through crosstalk, synergy and feedback mechanisms, they fine tune T cell signalling to match complex immune responses. In this article, we review the expression and function of various Ca^{2+}-permeable channels in the plasma membrane, endoplasmic reticulum, mitochondria and endolysosomes of T cells, and their role in shaping immunity and the pathogenesis of immune-mediated diseases.

Introduction

To mount effective immune responses, lymphocytes must transduce antigenic signals from the surface to their nuclei[1]. Signal transduction requires intracellular messenger molecules, the function of which can be switched on and off with time. For Ca^{2+} ions to acquire the on/off switch of a signalling molecule, their concentration within cellular microdomains must vary with time. As such, Ca^{2+} that reaches, for instance, the cytosol when cells are stimulated must subsequently exit into the extracellular space or be sequestered within the endoplasmic reticulum (ER) when signalling is discontinued. Due to their charge however,

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ions cannot diffuse freely across the lipid bilayer of biological membranes and instead require transmembrane channels and transporters to regulate Ca^{2+} concentrations within the cytosol and intracellular organelles[2].

T cells express several types of Ca^{2+}-permeable channels and transporters that control Ca^{2+} influx and efflux across the plasma membrane (PM)[1,3–5]. Intracellular organelles such as the ER, mitochondria and lysosomes also express specific channels and transporters that control Ca^{2+} release into the cytosol and Ca^{2+} uptake into these organelles[6–10] (FIG. 1). Ca^{2+} transport through channels is controlled by the concentration gradient of Ca^{2+} between each side of a biological membrane and by the electrical gradient or membrane potential **[G]** (Vm). The concentration gradient of Ca^{2+} across the PM favours a strong driving force of Ca^{2+} into the cytosol. T cells have a resting cytosolic Ca^{2+} concentration 50–100nM compared with the extracellular Ca^{2+} concentration which is 1–2mM[11]. The PM resting Vm in T cells is –60mV to –50mV, which also favours Ca^{2+} influx into the cytosol. Vm is determined by ion channels **[G]** conducting sodium (Na^+), potassium (K^+) and chloride (Cl^-), which indirectly regulate Ca^{2+} transport by controlling membrane potential[11]. These channels have been recently reviewed and are not discussed here[4].

In T cells, Ca^{2+} influx across the PM is mediated mainly through receptor-activated Ca^{2+} channels, which are operational at the resting Vm. Although the expression of PM voltage-activated Ca^{2+} (Ca_V) channels **[G]** is generally associated with excitable cells such as muscle cells and neurons, several Ca_V channels have been associated with T cell function[12–14]. An increase in cytosolic Ca^{2+} levels in T cells can also originate from the ER where Ca^{2+} concentrations are 300μM–1mM[15]. ER Ca^{2+} release channels include inositol-1,4,5-trisphosphate receptors **[G]** (IP_3Rs)[16] and ryanodine receptors **[G]** (RYRs)[17]. In turn, both Ca^{2+} entry and Ca^{2+} release channels couple receptor activation to metabolic activity and ATP production by providing cytosolic Ca^{2+} microdomains **[G]**, in the order of 1–10μM, for uptake by mitochondria through mitochondrial Ca^{2+} uniporter **[G]** (MCU)[7,18,19] (FIG. 1). The activities of three dehydrogenases in the tricarboxylic acid (TCA) cycle are regulated by mitochondrial Ca^{2+}[20–23], although this has not been shown specifically in T cells. Free mitochondrial Ca^{2+} concentration is estimated to vary between 0.1–10μM and can, in some instances of receptor stimulation, reach sub-millimolar levels[24].

Ion pumps **[G]** transport Ca^{2+} ions against the electrochemical gradient, thus requiring energy in the form of ATP. These ion pumps include PM Ca^{2+} ATPases (PMCAs)[25,26] and sarcoplasmic/ER Ca^{2+} ATPases (SERCAs)[27], which pump Ca^{2+} from the cytosol to the extracellular space and the ER, respectively (FIG. 1). PMCA4b, SERCA2b and SERCA3 are the major isoforms in lymphocytes[28,29]. Ca^{2+} efflux from mitochondria to the cytosol occurs via mitochondrial $Na^+/Ca^{2+}/Li^+$ exchanger (NCLX; also known as SLC24A6), a transporter that uses a Na^+ gradient to extrude mitochondrial Ca^{2+} [30–32] (FIG. 1). Ca^{2+} accumulation in endolysosomes and secretory vesicles occurs through the H^+/Ca^{2+} exchanger, which uses the steep proton concentration gradient to drive uptake of Ca^{2+} into these organelles[10,33]. Generation of high proton concentration in endolysosomes is a process that costs energy through the activity of vacuolar (V)-type H^+ ATPase[34]. Measurements of Ca^{2+} from lysosomes of macrophages suggest a concentration of 400–600μM, which varies depending on changes in pH within these organelles[35]. Ca^{2+} release from endolysosomes and secretory

vesicles into the cytosol occurs through two pore channels **[G]** (TPCs)[36] and transient receptor potential mucolipin (TRPML) channels **[G]**[33] and is believed to regulate exocytosis and endolysosomal fusion, trafficking and function[10,33,37]. Below, we discuss how Ca^{2+} microdomains generated by channels in the PM, ER, mitochondria and endolysosomes of T cells shape cellular Ca^{2+} signalling and immunity.

Plasma membrane Ca^{2+} channels

Antigen binding to the T cell receptor (TCR) initiates a cascade of protein phosphorylation that converges in the activation of phosphoinositide-specific phospholipase Cγ1 (PLCγ1)[38] (FIG. 1). PLCγ1 hydrolyzes, phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) into the soluble head group inositol-1,4,5-trisphosphate (IP_3) and the PM-associated lipid diacylglycerol (DAG)[6]. Diffusible IP_3 binds IP_3R on the ER to achieve two interdependent events: first, cause Ca^{2+} release into the cytosol through IP_3R[16]; and second, empty ER Ca^{2+} stores and thus indirectly activate Ca^{2+} influx across the PM through store-operated Ca^{2+} entry **[G]** (SOCE)[39,40] (FIG. 1). SOCE is mediated by PM channels activated as a consequence of emptying of ER Ca^{2+} stores. Inhibitors of SERCA pumps such as thapsigargin, which cause passive Ca^{2+} leak from the ER, activate SOCE to the same extent as receptor ligation[41]. Electrophysiological recordings of currents mediating SOCE identified an inwardly-rectifying Ca^{2+} selective current termed Ca^{2+} release-activated Ca^{2+} CRAC **[G]** [42].

Store-operated Ca^{2+} channels: activators

The molecular mechanisms coupling Ca^{2+} store depletion to activation of CRAC channels were discovered in the past decade[43,44]. The filling of ER stores is detected by the ER Ca^{2+} sensors stromal interaction molecule 1 (STIM1) and STIM2 in the ER membrane[45,46] (FIG. 1). When ER stores are depleted, Ca^{2+} is released from the Ca^{2+}-binding domain of STIMs, which triggers STIM aggregation and movement to junctional spaces where the ER and PM come into close contact, typically within 10–25nm. STIM2 is activated when modest store depletion occurs whereas STIM1 activation requires significant store depletion[47]. ORAI1 is the molecular correlate of PM CRAC channels, which was identified almost simultaneously by three independent groups[48–50]. ORAI1 and STIM1 are necessary and sufficient to support SOCE[48–55]. ORAI1 coalesces with STIMs in ER–PM junctions at the immune synapse **[G]** on store depletion-cell activation[56].

ORAI1.—The role of CRAC channels in T cell function was recognized long before the discovery of STIMs and ORAI. CRAC channels in T cells are primarily required for the activation of Ca^{2+}-dependent transcription factor isoforms of nuclear factor of activated T cells **[G]** (NFAT) (FIG. 2) and subsequent cytokine production, proliferation and immune competence[1,4,5]. However, the role of SOCE and CRAC in T cell function is more complex since *in vitro* addition of IL-2 to STIM1-deficient and ORAI1-deficient T cells from either mice or patients only partially restores TCR-mediated proliferation[57–60], suggesting additional roles for SOCE beyond the production of proliferative cytokines. One of the mechanisms by which SOCE and NFAT control T cell proliferation involve *de novo* transcription of other transcription factors and metabolic genes (discussed below)[61]. Further, SOCE synergizes with other pathways and depending on the magnitude, duration and nature

of Ca^{2+} signals (sustained versus oscillatory), a plethora of different transcription factors are activated[62] (FIG. 2).

T cells from patients suffering from a form of severe combined immunodeficiency (SCID) have normal Ca^{2+} release, whereas CRAC currents and SOCE were abrogated[59,63]. These forms of primary SCID are associated with impaired proliferation in response to mitogens due to alterations or mutations in either *ORAI1* or *STIM1* genes, most notably a single amino acid autosomal recessive mutation in ORAI1 (R91W), which generates an inactive channel[48,64]. Patients and mice deficient in either STIM1 or ORAI1 have increased susceptibility to bacterial, viral and fungal infections. In humans, immunodeficiency is accompanied by an autoimmune phenotype associated with lymphadenopathy, splenomegaly, thrombocytopenia and haemolytic anaemia that is presumably due to reduced numbers and altered suppressive function of $FOXP3^+$ regulatory T (T_{reg}) cells which depend on NFAT-mediated transcription[4]. An increasing number of patients with abrogated SOCE due to autosomal recessive mutations in *STIM1* and *ORAI1* were identified with strong reduction in numbers of T_{reg} cells and invariant natural killer T (iNKT) cells[64,65].

Most *Orai1*$^{-/-}$ and *Stim1*$^{-/-}$ mice on the C57BL/6 background die perinatally within 24h postpartum[66–68]. In contrast, *Stim2*$^{-/-}$[68], *Orai2*$^{-/-}$[69] and *Orai3*$^{-/-}$ (M. T., unpublished observations) mice are viable albeit *Stim2*$^{-/-}$ mice show slight growth retardation and die within 4–5 weeks after birth of unknown reasons. Cytokine production by $CD4^+$ and $CD8^+$ T cells from STIM1- and ORAI1-deficient patients and mice is dramatically impaired, including IFNγ, tumour necrosis factor (TNF), IL-2, IL-4, IL-10 and IL-17[4,5]. T cell-specific *Orai1* knockout mice show protection in the model experimental autoimmune encephalomyelitis (EAE) and have reduced T helper 1 (T_H1) and T_H17 cell-dependent cytokine production, including IL-17A and IFNγ[70,71]. Unlike the case in humans, *Orai1*$^{-/-}$ mice have no defect in differentiation and function of induced T_{reg} cells, which is likely due to the fact that SOCE is partially inhibited but not completely abrogated, reflecting a potential role for ORAI2 in mice versus humans. Unlike human T cells, SOCE in T cells from either *Orai1*$^{-/-}$ or ORAI1 R91W knock-in mice was only partially inhibited with a residual Ca^{2+} signal potentially reflecting ORAI2 involvement[67].

$CD4^+$ T cell-specific *Stim1* knockout mice have dramatically reduced SOCE in response to anti-CD3 and undetectable CRAC currents. These cells failed to sustain NFAT nuclear translocation and produce IL-2 on anti-CD3 and anti-CD28 co-stimulation[68]. STIM2 is a weaker ORAI1 activator compared with STIM1 and its activation occurs with slower kinetics [47,72]. It is thus not surprising that STIM2 cannot fully compensate for the lack of STIM1[4]. CRAC currents and SOCE in STIM2-deficient $CD4^+$ T cells was partially preserved but these cells failed to sustain higher plateaus of Ca^{2+} levels after 20min of TCR stimulation[4,5,68]. While NFAT nuclear translocation occurred after stimulation of *Stim2*$^{-/-}$ $CD4^+$ T cells, it was not sustained and IL-2 production was inhibited[68].

The phenotype of *Stim1*/*Stim2* $CD4^+$ T cell-specific double knockout (*Stim1*/*2*-dKO) mice is more severe, involving splenomegaly, lymphadenopathy and inflammation. They completely lack SOCE and show almost complete abrogation of IL-2 production[68]. *Stim1/2*-dKO mice develop humoral autoimmunity (at 6 months of age) including spontaneous

production of autoantibodies and altered differentiation of T follicular helper (T_{FH}) cells and T follicular regulatory (T_{FR}) cells. Indeed, STIM1 and STIM2 control the expression of the follicular T cell transcription factors IRF4, BATF and BCL-6 through SOCE activation of NFAT[73]. They showed impaired germinal centre reactions within B cell follicles, failed to produce virus-specific antibodies[73] and had 80–90% reduction in T_{reg} cell numbers in lymphoid organs with the few remaining T_{reg} cells presenting markedly impaired suppressive function[68]. Furthermore, differentiation of $CD4^+$ T cells into inducible $FOXP3^+$ T_{reg} cells and T_H17 cells are also impaired in *Stim1/2*-dKO mice[4]. The expression of glycolytic and oxidative phosphorylation enzymes as well as glucose transporters GLUT1 and GLUT3 were reduced in T cells from *Stim1/2*-dKO mice[61]. STIM1/2-mediated SOCE controls metabolic activity of T cells through calcineurin-mediated activation of NFAT and PI3K–mTOR signalling, which in turn regulate the expression of several transcription factors, including HIF1α, IRF4 and MYC, that are critical for expression of glycolytic enzymes[61]. However, the molecular mechanisms connecting Ca^{2+} to the activation of PI3K–AKT–mTOR signalling remain unknown.

Whereas STIM1- and ORAI1-mediated Ca^{2+} signals are crucial for T cell activation, proliferation and metabolism, excessive or prolonged Ca^{2+} signalling can lead to cell death. ORAI1 deficiency renders T cells resistant to death upon long-term exposure to anti-CD3 and anti-CD28, which coincided with severe reduction in nuclear import of NFAT[74]. Expression of either ORAI1 or constitutively active NFAT restored cell death in these cells. T cells from heterozygous *Orai1*$^{+/-}$ mice are not resistant to cell death, suggesting that the levels of cytosolic Ca^{2+} attained differentially activate distinct isoforms of NFAT[75]. NFAT2 is activated by moderate cytosolic Ca^{2+} levels and acts as a positive regulator of T cell proliferation, while NFAT1 activation requires high cytosolic Ca^{2+} and causes cell cycle arrest and death[76,77]. Resistance to death in *Orai1*$^{-/-}$ T cells correlated with reduced expression of death receptors and pro-apoptotic genes *Fasl* and *Pmaip1* (also known as *Noxa*)[74]. Similarly, *Stim1*$^{-/-}$ T cells were resistant to death on repeated TCR stimulation during chronic mice infection by *Mycobacterium tuberculosis*, with significantly decreased expression of the pro-apoptotic factors FAS, FASL and NOXA in the lungs[78]. Collectively, these results show that SOCE is the major Ca^{2+} entry pathway in T cells required for T cell immunity. STIM1/STIM2- and ORAI1-mediated SOCE is the central activator, which generates critical Ca^{2+} microdomains required for nuclear translocation of NFAT isoforms and optimal cytokine gene expression, metabolism and proliferation.

ORAI2.—*Orai2*$^{-/-}$ mice showed normal populations of thymocytes and peripheral lymphocytes and normal proportions of effector and memory T cells with largely maintained immune responses. ORAI2 was proposed as a negative modulator of ORAI1 that fine-tunes CRAC activity and the strength of T cell immunity[69]. Deletion of *Orai2* either globally or in $CD4^+$ T cells enhances SOCE only in naive T cells with no effect on effector T cells. By contrast, *Orai1*$^{-/-}$ mice show strong SOCE reduction in effector T cells and moderate reduction in naive T cells. In fact, both ORAI1 and ORAI2 contribute to CRAC channels in naive T cells where ORAI2 negatively modulates CRAC function. However, effector T cells downregulate ORAI2 and upregulate ORAI1, resulting in a major reduction of SOCE in effector T cells from *Orai1*$^{-/-}$ mice with marginal effects in the same cells from *Orai2*$^{-/-}$

mice[69]. Increased ORAI2 in naive T cells is expected to yield heteromeric CRAC channels with enhanced Ca^{2+}-dependent inactivation (CDI) that could control antigen-mediated signalling in cases of non-specific pathophysiological activation of naive T cells. In effector T cells however, strong ORAI1-mediated SOCE would allow for robust and immediate T cell activation in response to specific antigens. Consistent with a negative modulation of SOCE by ORAI2, an independent group reported that ORAI2 limits SOCE and degranulation in mast cells[79].

$CD4^+$ T cell-specific *Orai1*/*Orai2* double knockout (*Orai1/2*-dKO) mice largely recapitulate the phenotype of *Stim1/2*-dKO mice. They show no developmental defects of conventional $TCR\alpha\beta^+$ T cells while displaying splenomegaly and lymphadenopathy with increased proportions of immune cell subsets, including memory and effector T cells[69]. Similar to *Stim1/2*-dKO mice, altered immune homeostasis in peripheral lymphoid organs of *Orai1/2*-dKO mice could be explained by reduced T_{reg} cell numbers in the thymus and peripheral lymphoid organs[69]. Further, germinal centre formation, T_{FH} cell differentiation and antibody production in response to viral infection were significantly reduced in *Orai1/2*-dKO mice[69]. T cells from *Orai1/2*-dKO mice show dramatically inhibited proliferation in response to anti-CD3 and anti-CD28 co-stimulation. IL-2 only partially restored proliferation, reflecting the IL-2-independent role of CRAC in T cell metabolism discussed above[69]. Cytokine production was significantly hampered in T_H1, T_H17 and T_H2 cells from *Orai1/2*-dKO mice. In contrast, T cells from *Orai2*$^{-/-}$ mice showed largely preserved cytokine production[69]. Unlike wild-type mice and *Orai2*$^{-/-}$ mice, *Orai1/2*-dKO mice are protected from T cell-mediated autoimmunity and alloimmunity in adoptive transfer models of inflammatory bowel disease (IBD) and graft-versus-host disease (GvHD)[69]. Since ORAI2 is a negative regulator of SOCE, one might expect to see exacerbated immune responses in *Orai2*$^{-/-}$ mice in the IBD and GvHD models, which was not the case. The authors speculated that it is likely due to low ORAI2 expression in effector T cells, which largely mediate immunopathology in the colitis and GvHD models[69].

ORAI3.—Although ORAI3 in heteromeric associations with ORAI1 forms a store-independent Ca^{2+} selective channel in other cell types[80–84], the role of ORAI3 in T cells remains largely obscure. Unlike ORAI1, ORAI3 channels are resistant to inhibitory oxidation by hydrogen peroxide (H_2O_2)[85]. The equivalent of a reactive cysteine located in the second extracellular loop (Cys195) of ORAI1 is a glycine in ORAI3. Differentiation of $CD4^+$ T cells into effector T cells was accompanied by progressive loss of sensitivity to H_2O_2 and increased expression of ORAI3, suggesting this switch could allow effector cells to continue to proliferate and secrete cytokines in an oxidant-rich inflammatory milieu[85]. Knockdown of *Orai3* in a mouse model of collagen-induced arthritis slightly inhibited SOCE in splenocytes and thymocytes while reducing the production of inflammatory cytokines in joint protein extracts and serum and reducing the severity of arthritis[86].

Transient receptor potential channels: regulators

Transient receptor potential cation channel subfamily C, member 1 (TRPC1) and TRPC3 are expressed in T cells and knockdown of *TRPC3* caused a small reduction in SOCE and T cell proliferation[87]. Altered TRPC3 expression was reported in several T cell mutants with

defective Ca^{2+} entry in response to TCR ligation and introduction of *TRPC3* cDNA restored TCR-mediated Ca^{2+} influx[88]. The interpretation of these results is unclear and the involvement of TRPC3 in T cells remains highly contentious.

TRPM2 is expressed in T cells and is upregulated upon TCR ligation[89]. Although there is no evidence that TCR ligation activates TRPM2 in T cells, native TRPM2-mediated currents in Jurkat T cells can be activated by cyclic ADP ribose (cADPR), ADPR and NAADP[89]. TCR stimulation causes sustained increases in intracellular cADPR, and an antagonist of cADPR inhibited T cell activation and proliferation in response to TCR stimulation[90]. These results however can be explained by an effect of cADPR on RYR (discussed below). *Trpm2*$^{-/-}$ mice are partially protected in the EAE model and this correlates with reduced proliferation of $CD4^+$ T cells and inhibited secretion of IL-17 and IFNγ in response to TCR stimulation[91].

Increased cytosolic Ca^{2+} levels through SOCE upon TCR activation triggers Na^+ influx through TRPM4 (Kd ~400nM) to depolarize the PM and restrict further Ca^{2+} entry[92]. Ca^{2+}-induced activation of TRPM4 channels in T cells serves as a negative feedback mechanism, preventing excessive cytosolic Ca^{2+}-mediated toxicity and fine-tuning T cell responses[1,92,93]. Indeed, in the absence of TRPM4 channels, SOCE-mediated intracellular Ca^{2+} oscillations in response to stimulation of T cells by mitogens became sustained, resulting in enhanced cytosolic Ca^{2+} and increased IL-2 production[93]. The expression levels of TRPM4 channels seem to be higher in T_H2 cells than T_H1 cells, which could explain the reduced TCR-mediated Ca^{2+} flux in T_H2 cells[94], and might explain the relatively higher sensitivity to FAS-dependent apoptosis in T_H1 cells than T_H2 cells[95]. In contrast to TRPM4, Ca^{2+}-activated and voltage-activated K^+ channels counteract TRPM4 and play a positive modulatory role in T cell Ca^{2+} signalling. K^+ efflux, which maintains a hyperpolarized PM Vm and enhances the driving force for Ca^{2+} entry, is a crucial regulator of T cell immunity[96].

TRPM7 is critical for T cell development as T cell-specific *Trpm7* knockout mice have reduced numbers of peripheral T cells due to halting of thymocyte development at the double negative $CD4^-CD8^-$ stage[97]. Surprisingly, although these mice have a marked reduction in TRPM7 currents in thymocytes, acute Mg^{2+} uptake and total cellular Mg^{2+} levels were normal, suggesting that TRPM7 function in T cells likely occurs through either conduction of Ca^{2+} ions or its cytosolic serine/threonine kinase[97]. Notwithstanding a potential direct regulation of cytosolic Ca^{2+} signalling, TRPM7 might indirectly regulate SOCE in lymphocytes[98] (FIG. 3). Deletion of TRPM7 or its pharmacological inhibition inhibits SOCE. The positive regulation of SOCE by TRPM7 seems to require the kinase activity, but not the channel domain of TRPM7[98]. Thymocyte development was unaffected in TRPM7 kinase-dead (K1646R) knock-in mice, suggesting that the channel domain, but not the kinase domain, is crucial for T cell development[99]. Splenic T cells from TRPM7-K1646R knock-in mice exhibited normal TRPM7 currents while showing reduced SOCE and reduced proliferation with mice exhibiting splenomegaly and altered blastogenesis in response to co-stimulation with anti-CD3 and anti-CD28 antibodies or phorbol myristate acetate (PMA) and ionomycin[99]. An independent study showed that TRPM7-K1646R mice have normal T cell development, but TGFβ-mediated expression of CD103, SMAD2 phosphorylation and nuclear translocation and T_H17 cell differentiation were altered[100].

TRPM7 kinase activity was required for gut colonization by alloreactive T cells during an acute model of GvHD[100]. The TRPM7 channel domain, but not its kinase domain, regulates T cell homeostasis by mediating FAS-dependent T cell apoptosis through caspase activation[101].

One group reported the expression of TRPV1 in $CD4^+$ T cells[102]. These investigators reported a small inhibition of TCR-activated Ca^{2+} entry in TRPV1-deficient $CD4^+$ T cells but only under low extracellular Ca^{2+} concentrations with no effect on SOCE activated by thapsigargin. These authors suggested that TRPV1 is activated downstream of TCR through TRPV1 phosphorylation by the tyrosine kinase LCK[102]. However, this study lacks thorough electrophysiological characterization supporting TRPV1 activation downstream of TCR. Mice with TRPV1-deficient $CD4^+$ T cells showed reduced colitis severity during IBD[102] and were protected from allergic rhinitis[103]. The same group reported that TRPA1 inhibits TRPV1 through TRPA1–TRPV1 heteromultimeric assemblies to limit T cell activation and colitis in IBD[104]. Nevertheless, in light of complete abrogation of TCR-mediated Ca^{2+} signals in *Stim1/2*-dKO, the involvement of TRPV1 and TRPA1 (or TRPC3) in TCR-mediated Ca^{2+} signalling is a highly contentious issue and stronger electrophysiological evidence is required to support a role for these channels in T cell Ca^{2+} signalling. In summary, TRP channel isoforms are important regulators of TCR signalling and can exert this function by controlling the driving force for SOCE through Na^+ entry-mediated control of PM Vm (TRPM4), by regulating the activity of SOCE (TRPM7 kinase activity), or by potentially providing Ca^{2+} microdomains that either regulate T cell development or synergize with SOCE during T cell activation.

Purinergic ionotropic receptors: amplifiers

Purinergic ionotropic receptors (P2RX) represent a family of seven nonselective trimeric channels with a multitude of splice variants capable of homomerization and heteromerization[105]. P2RX channels conduct Na^+ and Ca^{2+} and are directly activated by extracellular ATP. Most P2RX isoforms are expressed in thymocytes and peripheral T cells[106]. However, electrophysiological characterization of P2RX receptors in T cells is lacking and the precise ions (Ca^{2+} versus Na^+ entry or K^+ efflux) mediating P2RX receptor effects are unclear. While knockout mice for either *P2rx1*, *P2rx4* or *P2rx7* have normal T cell development, one group reported that *P2rx2/P2rx3* double knockout mice exhibit splenomegaly[107], with a different group reporting reduced cellular numbers in the thymus and bone marrow and altered lymph follicles in spleen and peripheral lymph nodes[108], suggesting isoform redundancy. Very little is known about P2RX5 and P2RX6, except that the expression of P2RX5 is increased in activated T cells[109]. Based on small interfering RNA-mediated knockdown of *P2RX1*, *P2RX4* and *P2RX7* in primary T cells and Jurkat T cells, these channels seem to couple to activation of NFAT-dependent IL-2 production and T cell proliferation in response to TCR ligation[106]. TCR-mediated activation of P2RX receptors could presumably be initiated by IP_3R-dependent and SOCE-dependent mitochondrial Ca^{2+} uptake leading to the production of ATP and its extracellular export to activate P2RX receptors in an autocrine and paracrine fashion (FIG. 4). In support of this, Pannexin1 channels, which can export ATP to the extracellular space, translocate to the immune synapse where they colocalize with STIM1, ORAI1, P2RX1 and P2RX4[110]. In this

regard, P2RX receptors serve as amplifiers of TCR-mediated SOCE and T cell effector functions. TCR ligation is known to promote mitochondrial ATP production, which would fuel P2RX receptor-mediated Ca^{2+} entry to further enhance ATP production, thus amplifying weak TCR signals to produce calcineurin–NFAT activation and mount adequate T cell responses (FIG. 4).

P2RX7 was initially proposed to mediate lymphocyte cytotoxicity and cell death in response to enhanced extracellular ATP levels. However, under moderate conditions of stimulation, P2RX7 can promote T cell activation and growth by coupling to mitochondrial bioenergetics and ATP production (presumably by providing cytosolic Ca^{2+} for mitochondrial uptake) and by enhancing glycolysis through transcriptional upregulation of glycolytic enzymes[106]. Excessive P2RX7 stimulation causes mitochondrial Ca^{2+} overload, uncoupling of oxidative phosphorylation and cell death[106], which is analogous to TCR-mediated activation of SOCE where the strength of stimulation and the expression levels of STIM and ORAI can promote either T cell activation or death. Increasing evidence points towards a role for P2RX1, P2RX4 and P2RX7 in promoting T cell-mediated immunity and inflammatory responses in vivo, with P2RX7 being the most studied in this context[105,106]. A homeostatic role for P2RX1 was proposed to be required for T cell activation. This consists of a positive feedback loop involving basal mitochondrial ATP production, ATP release and activation of P2RX1[111]. Stimulated T cells in lymph nodes secrete ATP and activate P2RX4 and P2RX7 to mediate paracrine calcium waves in neighbouring resting lymphocytes to reduce their motility and help antigenic scanning of resident dendritic cells[112]. P2RX4 and P2RX7 seem to play an important role in the amplification of TCR-mediated Ca^{2+} signalling in non-conventional γδ T cell differentiation and cytokine production[113,114].

Pan inhibition of P2RX receptors in mice using oxidized ATP prevented the development of colitis in adoptive T cell transfer models and was associated with increased T_{reg} cell numbers in peripheral lymph nodes and reduced production of IL-17. The activation of the ubiquitous P2RX7 inhibits the immunosuppressive functions of T_{reg} cell and facilitates their conversion into T_H17 cells, which is accompanied by reduced expression of FOXP3 and enhanced expression of the T_H17 cell-specific transcription factor RORγt[115] (FIG. 4). Consistent with these findings, inactivation of P2RX7 with oxidized ATP showed preserved pancreatic islet allografts in mice and reduced T_H1 and T_H17 cells in this model[116]. Inflammatory sites contain high concentrations of ATP, in the order of hundred micromolar[106]. P2RX7 expression was specifically upregulated in graft-infiltrating lymphocytes in humans and mice with cardiac transplants and P2RX7 inhibition delayed coronaropathy and enhanced survival of cardiac allografts in a mouse model of chronic rejection[117]. In ischaemia-reperfusion injury, P2RX receptor inhibition by oxidized ATP promoted expansion of T_{reg} cells, and blunted acute renal injury and improved renal recovery[118]. *P2rx7*$^{-/-}$ mice are protected in the EAE model[119] and in a model of experimental autoimmune uveoretinitis[120]. *P2rx7*$^{-/-}$ mice have enhanced susceptibility to *Plasmodium chabaudi* malaria infection, which correlated with decreased IL-2 and IFNγ secretion by $CD4^+$ T cells, T-bet expression and impaired T_H1 cell differentiation. The requirement for P2RX7 in T_H1 cell differentiation during infection was accompanied by a reduction in the splenic T_{FH} cell population of infected mice[121]. Of note, the beneficial effects of P2RX7 inhibition on reducing inflammation through promotion of T_{reg} cells in the

digestive tract in a colitis model were offset by anti-apoptotic effects of P2RX7 blockade on intestinal epithelial cells, which caused increased proliferation of these cells to promote colitis-associated cancer[122]. Deletion or pharmacological inhibition of P2RX7 is known to enhance tumour metastasis and to drastically alter immunogenic cell death of cancers, presumably because P2RX7 is required for dendritic cells to release IL-1β (for review, see[106]).

Recent work showed that P2RX7 is required for generation and function of durable long-lived memory $CD8^+$ T cells but not short-lived effector $CD8^+$ T cells. P2RX7 achieves this role by promoting metabolic fitness of these cells through induction of AMP-activated protein kinase and maintenance of mitochondrial homeostasis and metabolism[123]. While overall data supports P2RX7 activity as pro-inflammatory, an earlier report showed that *P2rx7*$^{-/-}$ mice have exacerbated EAE due to reduced apoptosis of lymphocytes[124]. This could be explained by the *P2rx7*$^{-/-}$ strain used that lacks P2RX7 in macrophages and dendritic cells while unexpectedly expressing higher levels in T cells[125]. Nevertheless, there are conflicting reports showing that exogenous ATP suppresses T cell activation and IL-2 production[126] and ATP production by dying acute myeloid leukemia cells during chemotherapy causes immune suppression[127]. The overall data suggests the P2RX receptors play a homeostatic (P2RX1) and amplifying (P2RX4 and P2RX7) role of TCR signalling. P2RX receptors seem to synergize with SOCE through a positive feedback loop involving mitochondrial Ca^{2+} uptake and enhanced ATP production. SOCE-mediated mitochondrial ATP production, ATP export by pannexin1 channels and ATP action on P2RX receptors can cause further Ca^{2+} entry to amplify weak TCR signals and help in antigen scanning.

Ca_V channels: modulators

L-type Ca^{2+} channels.—Transcripts and proteins of all four members of the L-type (long-lasting) Ca_V channel family and regulatory β3 and β4 subunits are expressed in mouse and human T cells and their expression is increased upon anti-CD3 and anti-CD28 co-stimulation[128]. Yet, the involvement of voltage-activated Ca^{2+} channels in T cell function is a highly contentious issue. T cells rely on a negative membrane potential for Ca^{2+} entry and in which depolarization is expected to inhibit Ca^{2+} transport across the PM. Nevertheless, several studies reported that T cells express truncated or alternatively spliced forms of L-type Ca_V channels, which either lack one voltage sensor (e.g. $Ca_V1.4$) or a linker adjacent to voltage sensor ($Ca_V1.1$)[129], suggesting that Ca_V channels in T cells might not be voltage-gated and can be activated by alternative mechanisms. Such mechanisms may involve phosphorylation of Ca_V channels by protein kinase C (PKC) and protein kinase A (PKA); both kinases are activated downstream SOCE and are Ca_V regulators in different cell types[130] (FIG. 5). Of significance, constitutively active $Ca_V1.2$ channels, which required PKC, were reported in arterial smooth muscle cells[131–134]. Quantal Ca^{2+} sparklets and single $Ca_V1.2$ channel currents were recorded at membrane potentials as low as –90 mV[135]. Similar to its non-channel function in skeletal muscle[136], the $Ca_V1.1$ splice variant expressed in T cells[137] could be involved in a voltage-independent Ca^{2+} entry-independent conformational coupling with RYR at ER–PM junctions to induce ER Ca^{2+} release via RYR (discussed below) (FIG. 5). However, experimental data in support of conformational

coupling of $Ca_V1.1$ to RYR or $Ca_V1.2$ channel clustering and activation in lymphocytes is lacking.

One major limitation is the lack of thorough biophysical characterization of L-type currents in T cells by patch clamp electrophysiology **[G]** as any alteration of Ca^{2+} signalling measured with fluorescent dyes can be equally explained by changes in membrane potential. Although a small Ca_V-like current activated by depolarizing voltage ramps was reported in wild-type T cells and was lost in T cells from $Ca_V1.4$-deficient mice, these currents, which were recorded in non-physiological extracellular solutions containing high Ba^{2+} concentrations (100 mM) as the charge carrier, remain controversial[13]. Independent recordings from activated human T cells readily revealed CRAC currents but failed to detect voltage-activated Ca^{2+} currents, using the same conditions that produced voltage-activated Ca^{2+} currents in neuronal PC12 cells[56]. Ca^{2+} measurements in response to TCR stimulation or thapsigargin showed reduction in Ca^{2+} release and Ca^{2+} entry in $Ca_V1.4$-deficient T cells, leading the authors to propose that $Ca_V1.4$ mediates basal Ca^{2+} entry important for ER refilling[13] (FIG. 5). Whether loss of $Ca_V1.4$ in T cells alters the expression of ER and PM Ca^{2+} transport proteins or membrane potential was not explored. $Ca_V1.4$-deficient mice showed a reduction in mature thymocytes and in peripheral $CD4^+$ and $CD8^+$ T cells, suggesting a role for $Ca_V1.4$ in promoting T cell survival and expansion[13]. Similarly, knockout of the auxiliary subunits β3 and β4 in mice led to reduction in $Ca_V1.1$ expression and in partial inhibition of TCR-activated Ca^{2+} signals and NFAT1 and NFAT2 activation in $CD4^+$ T cells[12]. The β subunit-deficient $CD4^+$ T cells displayed defects in cytokine production (IL-2, IL-4 and IFNγ) but proliferation in response to TCR stimulation was unaffected[12]. Deletion of the β3 subunit led to loss of $Ca_V1.4$ expression, impaired TCR-activated Ca^{2+} influx and inhibited survival of $CD8^+$ T cells[138]. T cell-specific deletion of the β2 subunit reduced the expression of $Ca_V1.2$ and $Ca_V1.3$ and altered T cell development, causing a dramatic decrease in thymocytes and peripheral T cell numbers[139]. $Ca_V1.2$ and $Ca_V1.3$ were proposed to be crucial for T_H2 cells (but not for T_H1 cells); their inhibition was protective in experimental models of asthma[129].

T-type Ca^{2+} channels.—The α1 pore-forming subunit of $Ca_V3.1$ is expressed at the PM of $CD4^+$ T cells[14]. Notwithstanding a potential activation of T-type (transient) Ca^{2+} channels through voltage-independent mechanisms, robust T-type currents were readily apparent in T cells at resting Vm and had a range of window currents from –65mV to –25mV, which is compatible with T-type channels being functional under resting membrane potential in T cells[14] (FIG. 5). Knockout of the $Ca_V3.1$ α1 subunit in mice led to a ~50% reduction of T-type currents in T cells, with the remaining current likely reflecting compensatory upregulation of $Ca_V3.2$ and $Ca_V3.3$ channels[14]. T cells from $Ca_V3.1$-deficient mice showed normal TCR-activated Ca^{2+} entry, suggesting that $Ca_V3.1$ mediates constitutive Ca^{2+} entry independently of SOCE. Naive $CD4^+$ T cells from $Ca_V3.1$-deficient mice as well as T cells with acute deletion of $Ca_V3.1$ and polarized in vitro into T_H17 cells showed decreased IL-17A, IL-17F and IL-21 production[14]. $Ca_V3.1$-deficient mice were protected from EAE and showed inhibited production of granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine implicated in susceptibility to EAE, by brain-infiltrating T_H1 and T_H17 cells[14]. Nuclear translocation of NFAT1 and NFAT2 after anti-CD3 and anti-CD28 co-

stimulation was reduced in $Ca_V3.1$-deficient $CD4^+$ T cells, suggesting that basal $Ca_V3.1$-mediated Ca^{2+} increase is crucial for calcineurin–NFAT activation to drive transcription of cytokines and other transcription factors, including GM-CSF and RORγt[14] (FIG. 5).

Regardless of how L-type and T-type channel isoforms are activated in T cells, they likely provide discrete Ca^{2+} microdomains that fine-tune TCR-mediated Ca^{2+} signals. This can be achieved through coupling of Ca^{2+} microdomains to SERCA for ER Ca^{2+} store refilling and optimal SOCE function, by directly sustaining NFAT activation and production of cytokines during T cell activation, or by coupling to downstream pathways that control differentiation of specific T cell populations (such as T_H17 cells).

ER Ca^{2+} release channels: initiators and orchestrators

ER Ca^{2+} release channels provide initial cytosolic Ca^{2+} microdomains in response to TCR ligation. They function through this primary mode to stimulate further ER Ca^{2+} release and Ca^{2+} entry across the PM required for proliferation and secretion of cytokines and provide Ca^{2+} microdomains for uptake by mitochondria to enhance T cell metabolism and support activation. In the long term, ER Ca^{2+} release channels can have a sustaining and synergistic effect on cellular Ca^{2+} signals emanating from PM and organellar Ca^{2+} channels.

IP_3Rs.—The initial step in response to TCR stimulation, preceding Ca^{2+} entry across the PM, is Ca^{2+} release from the ER by soluble second messengers. The most extensively studied Ca^{2+}-releasing second messenger is IP_3, which causes Ca^{2+} release through IP_3R[6]. T cells express all three isoforms of IP_3R, which have distinct sensitivity to IP_3, allosteric regulation by Ca^{2+} and Ca^{2+} release patterns[8]. Heteromerization of different IP_3R isoforms can further enhance the diversity of Ca^{2+} release signals[140]. Type I IP_3R (IP_3R1) is essential for IP_3-mediated Ca^{2+} release in response to TCR stimulation[141]. T cells deficient in IP_3R1 are resistant to apoptosis induced by TCR ligation, dexamethasone and FAS stimulation[142]. Recently, a Tie2-Cre mouse was used to conditionally delete all three genes encoding IP_3R isoforms in mice. In IP_3R triple knockout mice, thymocyte development was arrested at the double negative 4 (DN4; $CD44^-CD25^-$) and immature single positive (ISP) stages, which coincided with the development of T cell acute lymphoblastic leukemia (T-ALL) in knockout mice[143]. Thymocyte development was normal up to the $CD4^+CD8^+$ double positive (DP) stage when one or two IP_3R types were deleted, highlighting IP_3R redundancy in T cell function[143]. Mechanistically, IP_3R triple knockout leads to maintained expression of SOX13 past the DN3 stage. SOX13 is an antagonist of the WNT transcription factor TCF7 (also known as TCF1). The continued expression of SOX13 inhibits TCF7 and halts thymocyte development at the DN to DP transition[143]. IP_3R-mediated Ca^{2+} signals were also proposed to inhibit oncogenic Notch1 transcriptional target HES1 to promote tumorigenesis[143].

RYRs.—Guse and co-workers proposed a role for the second messengers NAADP and cADPR in T cell Ca^{2+} signalling. NAADP and cADPR, which act on RYR Ca^{2+} release channels, are produced after TCR ligation[17,90]. NAADP, which is produced early after TCR stimulation, provides a trigger by targeting RYR1 through binding to an unknown accessory molecule[144]. Early (within 20 milliseconds) Ca^{2+} microdomains near the PM following

activation by either TCR ligation or NAADP injection were absent or markedly reduced in T cells from *Ryr1*$^{-/-}$ mice[145]. This early Ca^{2+} signal facilitates the activation of IP_3R by IP_3 within seconds of TCR stimulation through the process of Ca^{2+}-induced Ca^{2+} release (FIG. 6). Later, within tens of minutes, production of cADPR would act on RYR2 or RYR3 to sustain Ca^{2+} signalling for an extended period of time[17,144]. In T cells, production of IP_3 and cADPR occur within tens of seconds and minutes respectively, and were proposed to sustain late phases of Ca^{2+} signalling[146] (FIG. 6). However, although the role of IP_3 in sustaining Ca^{2+} signalling is well established, the involvement of cADPR is less certain.

A pharmacological compound that blocks the interaction of NAADP with RYR1 inhibited NAADP-mediated Ca^{2+} release, formation of the immune synapse, nuclear translocation of NFAT, IL-2 production and proliferation of $CD4^+$ T cells[147]. However, pharmacological inhibition of RYR by ryanodine suggested that Ca^{2+} release via RYR was not the direct result of TCR activation but rather depended on RYR activation by Ca^{2+} originating from SOCE[148]. In turn, Ca^{2+} release via RYR maintains proximal ER store depletion to sustain SOCE[149] (FIG. 6). Inhibition of RYR reduced T cell proliferation and IL-2 production[148,149]. However, an independent study showed that T cells lacking RYR3, which is the main isoform in T cells, have normal proliferation to mitogens[150]. Furthermore, depending on the cell type considered, the channel (or channels) targeted by NAADP remain uncertain. Some studies suggested that NAADP activates the endolysosomal channels, TPCs[151], with potential significance for the function of cytotoxic T cells (discussed below). Other studies showed Ca^{2+} release from the ER in response to NAADP but failed to observe activation of Ca^{2+} release from acidic organelles[152], whereas independent studies showed that NAADP released Ca^{2+} from both ER and acidic organelles[153].

Mitochondrial Ca^{2+} signalling

Mitochondria control biosynthesis, thermogenesis, ATP and reactive oxygen species (ROS) production and cell death through apoptosis[154]. Mitochondria are active participants in Ca^{2+} signalling[7,155–158]. They uptake cytosolic Ca^{2+} to enhance bioenergetics necessary for T cell activation and proliferation but can also act as buffers and thus shape the spatial distribution of cytosolic Ca^{2+} signals[7,155–158] (FIG. 6). In T cells, mitochondria move towards the immune synapse, during interactions between T cells and antigen-presenting cells[159,160]. ORAI1 channels in T cells are also located at the immune synapse and Ca^{2+} buffering by mitochondria within close vicinity of ORAI1 relieves the channel's CDI enabling cells to maintain sustained levels of Ca^{2+} necessary for efficient NFAT activation[161,162]. While mitochondria clearly cannot fit within the small space of the ER-PM junctions *per se*, they locate as close as ~200nm from the PM[163]. Intriguingly, at the immune synapse mitochondria regulate PMCA[164]. Typically, PMCA is inhibited at resting Ca^{2+} levels but as cytosolic Ca^{2+} increases, its activity slowly increases due to binding of Ca^{2+}-calmodulin to its C-terminus, a process known as modulation, which allows fast Ca^{2+} transients to proceed relatively unaffected while sustained Ca^{2+} signals that might be toxic to cells are curtailed. During immune synapse formation, PMCA4b, the most common isoform of PMCA in T cells, redistributes into areas that are juxtaposed with mitochondria[164]. This close apposition allows mitochondria to buffer Ca^{2+} away from PMCA4b, preventing its modulation and reducing Ca^{2+} extrusion outside the cell (FIG. 6), thus maximizing ORAI1-mediated Ca^{2+}

signalling for robust T cell activation[164]. Further, STIM1 physically interacts with PMCA at the IS to inhibit PMCA-mediated Ca^{2+} clearance during T cell activation[165].

Mitochondrial Ca^{2+} uptake through the inner membrane channel MCU[18,19] is driven by the steep mitochondrial membrane potential (~−180 mV) whereas mitochondrial Ca^{2+} extrusion is achieved mainly by NCLX[30,158,166] and potentially by H^+/Ca^{2+} exchange activity[167]. Mitochondrial Ca^{2+} extrusion can shuttle Ca^{2+} to different areas of the cytosol and support efficient ER refilling by dumping Ca^{2+} at the vicinity of SERCA pumps[168]. Sustained elevation of cytosolic Ca^{2+} in response to TCR stimulation requires not only mitochondrial Ca^{2+} uptake but also depends on mitochondrial Ca^{2+} extrusion. Inhibition of mitochondrial Ca^{2+} extrusion in T cells through Na^+ depletion inhibits TCR- and store depletion-activated Ca^{2+} entry[161]. Na^+ depletion or NCLX deficiency prevents mitochondrial Ca^{2+} extrusion, cause mitochondrial Ca^{2+} overload and enhance mitochondrial ROS, which cause ORAI1 oxidation at Cys195 and its inhibition[31]. The role of MCU and NCLX in T cell Ca^{2+} signalling and function remain essentially unknown.

Vesicular Ca^{2+} channels

Trafficking by means of endosomes, recycling endosomes, lysosomes and secretory lysosomes is essential for T cell function[169,170]. Ca^{2+} release from these organelles has emerged as crucial for T cell activation[169]. Recycling endosomes can sustain TCR signalling at the immune synapse through endocytosis of exhausted signalling molecules and replenishment of endocytosed molecules, including TCR–CD3 complexes[171]. Guided secretion of cytokines and lytic enzymes at the immune synapse also occurs through secretory vesicles, exosomes and secretory lysosomes. The best studied are lysosomes or lysosome-like acidic organelles where Ca^{2+} release was proposed to occur through NAADP action on TPCs[172–174]. TPC2 is found in lysosomes whereas TPC1 is predominantly expressed in endosomes[175]. Whether TPCs are Ca^{2+} permeable channels is a contentious issue since whole-endolysosome patch clamp measurements suggested that TPCs that are activated by $PI(3,5)P_2$ are Na^+-selective[176,177]. Unlike $PI(4,5)P_2$, which is associated with PM, $PI(3,5)P_2$ is enriched in late endosomes and lysosomes[178]. TPCs play a crucial role in vesicular trafficking associated with endocytosis and autophagy[10]. Upon cytotoxic T cell activation, NAADP-mediated activation of TPCs cause Ca^{2+} release from cytolytic granules, migration of these granules to the immune synapse and exocytosis of perforin and granzymes in the synaptic space to induce target cell killing[179]. These TPC functions could not be recapitulated by increasing global cytosolic Ca^{2+} with either a cell permeant IP_3 or ionomycin, suggesting that IP_3-mediated Ca^{2+} release and SOCE (which are crucial for exocytosis) are not sufficient and additional Ca^{2+} microdomains at the outer surface of secretory lysosomes are required[179].

Mucolipin TRPs (TRPML1–TRPML3) are non-selective Ca^{2+}-permeable channels found both at the PM and in endolysosomes[33]. TRPML1 is ubiquitously expressed throughout the body whereas TRPML2 and TRPML3 are more tissue-restricted with TRPML2 prominently expressed in immune cells[180,181]. TRPML1 is enriched on the lysosomal surface, whereas TRPML2 is localized mainly to endosomes[182]. TRPML3 is found on lysosomes and on the PM[181,183]. Similar to TPCs, the activation of mucolipin TRPs depends on $PI(3,5)P_2$[184].

TRPML1 is required for vesicular fusion, trafficking of endocytic vesicles, lysosomal exocytosis and phagocytosis[185,186].

Intriguingly, TRPML channels are inhibited by PM-localized $PI(4,5)P_2$, suggesting that TRPMLs function is restricted to endolysosomes[187,188]. Adenosine in the lysosomal lumen was also reported to be a TRPML1 inhibitor. Abnormally increased adenosine within lysosomes (normally generated from nucleic acids and ATP) impairs the function of TRPML1 channels leading to dysfunctional, enlarged and alkaline lysosomes and altered lymphocyte function[189]. Increased lysosomal adenosine in patients deficient in adenosine deaminase have impaired immune function reminiscent of SCID[190].

Summary and perspectives

T cells express a plethora of Ca^{2+} permeable channels at various locations, with unique activation mechanisms that are necessary for T cell activation, maturation and secretion of cytokines. Ca^{2+} signalling in lymphocytes is tightly coupled to the regulation of adaptive immunity, differentiation and metabolism. Future research should tackle this increasingly diverse array of Ca^{2+} channels, their regulation, temporal expression, unique functions and how they synergize in controlling specific aspects of immunity. Why do cells require this large arsenal of channels to conduct the same ion? Distinct channels positioned in discrete locations within the PM, mitochondria and other organelles sculpt a dynamic cytosolic "Ca^{2+} signature" in the form of Ca^{2+} microdomains at the vicinity of key Ca^{2+}-activated effector proteins. Studies showed that Ca^{2+} concentrations reach hundreds of μM nanometers away from a channel pore, while few μm away from the pore Ca^{2+} concentrations precipitously fall to the nM range[191]. Because Ca^{2+} signals can poorly diffuse through cellular buffers, highly localized spatial and temporal Ca^{2+} microdomains for Ca^{2+} signalling and Ca^{2+} transfer between different organelles are exquisitely specific regulators of T cell function. Ca^{2+} is transferred between organelles through highly-specialized and tight contact sites, epitomized by the mitochondria-associated membranes (MAMs), which are intimate ER-mitochondria contact regions. Therefore, the source and location of a Ca^{2+} signal is what determines which downstream signalling pathways and gene programs are activated, and it is the major determinant by which the ubiquitous Ca^{2+} signal achieves specificity.

Depending on the nature of stimuli, their concentration and affinity for receptors, Ca^{2+} signals can take the shape of either sustained plateaus or regenerative oscillations. While it is appreciated that the strength and duration of Ca^{2+} plateaus can determine whether a T cell proliferates or dies, different levels and durations of Ca^{2+} plateaus likely activate different transcription programs in a T cell subset-dependent manner. The frequency and amplitude of Ca^{2+} oscillations, which are controlled by factors such as the strength of stimulation and levels of ion channels (e.g. TRPM4), provide a bandwidth of digitally distinct signals that are decoded by downstream cytosolic and nuclear proteins. It is therefore not surprising that a large number of channel isoforms are required to mount effective immune responses. To ensure a high degree of specificity, T cells probably require few "initiating" Ca^{2+} signals (for example, through IP_3R) and "activating" Ca^{2+} signals (for example, through ORAI1), as well as many modulatory Ca^{2+} signals (for example, through Ca_V, P2RX and TRP), for fine

tuning T cell activation. This incredibly complex network of channels provides the necessary diversity and flexibility to match the staggering complexity of immune responses.

While evidence exists for Ca^{2+} transfer from ER and PM to mitochondria, potential sites for Ca^{2+} transfer between endolysosomes and mitochondria are unknown. The field of Ca^{2+} signalling by intracellular vesicles is at its infancy with even scarcer knowledge as it relates to immune cells. The contribution of Ca^{2+} release from organelles to T cell activation and the potential differences in the patterns of expression of specific vesicular Ca^{2+} channels in different T cell subsets await future investigations. The advent of super-resolution imaging is beginning to allow for dynamic measurements of Ca^{2+} microdomains in living T cells. The development of cells and mice with tissue-specific expression of genetically-encoded Ca^{2+} indicators with custom-made dissociation constants for Ca^{2+} that can be targeted to specific PM areas, different sides of organellar membranes or even tethered to channels will likely speed up our understanding of the contributions of different channels to T cell function. In any case, the era of studying Ca^{2+} signalling in a specific cellular compartment in isolation is coming to an end. Ca^{2+} signalling operates as a network and regulates what was long considered housekeeping functions, such as metabolism. Future studies should consider simultaneous measurements of Ca^{2+} in different organelles, something that is now possible. As we toil to better understand Ca^{2+} signalling in T cells, we ought to adapt inclusive approaches that addresses not only signalling and gene transcription, but also metabolism, organellar trafficking and secretion for all these converge to exert crucial control over T cell activation and immune function. This understanding will lead to specific channel inhibitors for therapy of transplant rejection, immune disease and inflammatory disease.

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Glossary

Ca^{2+} microdomains
Discrete sites in the cytosol of cells localized near the mouth (within few nm) of Ca^{2+} channels of either plasma membrane or organellar membranes. These regions, which contain high Ca^{2+} concentrations, are the sites where specific Ca^{2+}-activated effector proteins are located. Ca^{2+} microdomains near specific Ca^{2+} channels are the major means by which the ubiquitous Ca^{2+} ion ensures specificity of signal transduction

Membrane potential
The difference in electric potential between the interior and the exterior of a biological membrane. In resting T cells, the plasma membrane potential is typically between –60 and –50 mV

Ion channels
Transmembrane proteins that form oligomers around a central pore, which allows specific ions to flow across biological membranes. Channels conduct ions according to the

electrochemical gradient of this membrane and therefore this process does not consume energy in the form of ATP

Voltage-activated Ca^{2+} (Ca_V) channels

Ca^{2+} selective channels located at the plasma membrane (PM) of excitable cells, such as muscle cells and neurons, and activated in response to PM depolarization. In T cells Ca_V channels might be activated by voltage-dependent or voltage-independent means

Inositol-1,4,5-trisphosphate receptors

Ca^{2+} release channels present in the endoplasmic reticulum (ER) membrane that release Ca^{2+} from the ER lumen to the cytosol in response to allosteric binding of Ca^{2+} and inositol-1,4,5-trisphosphate

Ryanodine receptors

Ca^{2+} release channels present in the endoplasmic reticulum (ER) membrane that mediate release of Ca^{2+} from the ER lumen to the cytosol on activation by Ca^{2+}, nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (cADPR)

Mitochondrial Ca^{2+} uniporter

A mitochondrial Ca^{2+} selective channel complex located in the inner mitochondrial membrane that conducts Ca^{2+} from the cytosol to the mitochondrial matrix

Ion pumps

Transmembrane proteins that transport ions against the electrochemical gradient of a membrane and this function requires energy in the form of ATP hydrolysis. Examples include: sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), which pumps Ca^{2+} from the cytosol into the ER; and plasma membrane Ca^{2+} ATPase (PMCA), which pumps Ca^{2+} from the cytosol to the extracellular space

Two pore channels

Ion channels located in the membrane of endolysosomes and proposed to release Ca^{2+} and Na^{+} from endolysosomes to the cytosol and are activated by NAADP and by phosphoinositide species localized in the endolysosomal membrane, such as phosphatidylinositol-3,5-bisphosphate

Transient receptor potential mucolipin (TRPML) channels

Non-selective cation channels located on the surface of endolysosomes that release Ca^{2+} and Na^{+} from these organelles into the cytosol. TRPML channels are activated by phosphoinositide species localized in the endolysosomal membrane such as phosphatidylinositol-3,5-bisphosphate

Store-operated Ca^{2+} entry

The most ubiquitous Ca^{2+} influx pathway in non-excitable cells, which is activated when endoplasmic reticulum Ca^{2+} stores are depleted. It is mediated by plasma membrane ORAI Ca^{2+} channels activated by direct binding of stromal interaction molecule (STIM) proteins

Ca^{2+} release-activated Ca^{2+} (CRAC)

The biophysical manifestation of store-operated Ca^{2+} entry and ORAI channels measured by whole-cell patch clamp electrophysiology. CRAC currents are highly Ca^{2+}-selective

Nuclear factor of activated T cells (NFAT)
An important family of transcription factors that are Ca^{2+}-activated. Ca^{2+}–calmodulin activates the phosphatase calcineurin, which then dephosphorylates NFAT causing its import into the nucleus to mediate gene transcription of many cytokines, transcription factors and metabolic genes

Immune synapse
The nanoscale interface of interaction between a lymphocyte and an antigen presenting cell

Patch clamp electrophysiology
A laboratory technique used to measure ionic currents through specific channels from single living cells or from a patch of cell membrane. Under the voltage clamp configuration, controlled (clamped) voltage values are applied to the cell membrane by the experimenter and the resulting currents are measured

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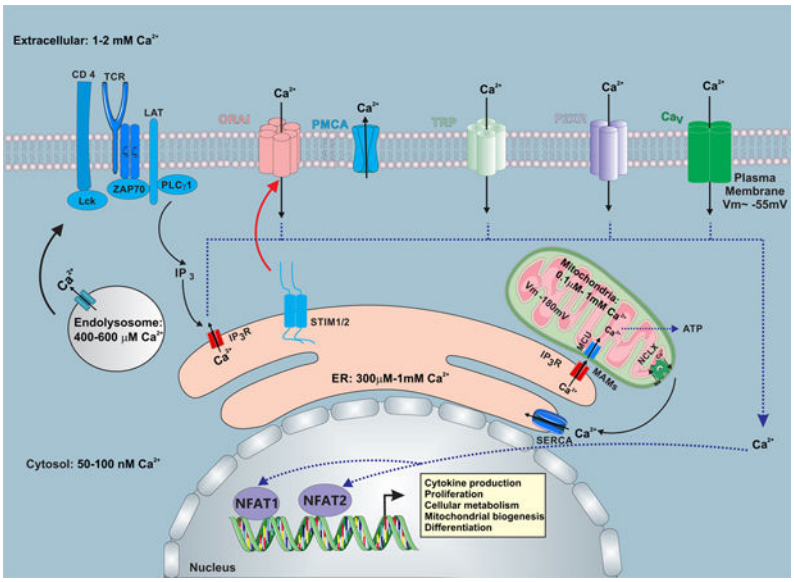

Figure 1 |. Calcium signalling in T cells.

Stimulation of the T cell receptor (TCR) by specific antigens leads to activation of phospholipase Cγ1 (PLCγ1), the production of inositol-1,4,5-trisphosphate (IP_3) and Ca^{2+} release from endoplasmic reticulum (ER) Ca^{2+} stores via IP_3 receptor (IP_3R) channels. The decrease in Ca^{2+} levels within the ER lumen is sensed by low affinity EF-hands of stromal interaction molecule 1 (STIM1) and STIM2. STIM proteins gain an extended conformation to trap and activate ORAI1 proteins at the plasma membrane (PM) and induce store operated Ca^{2+} entry (SOCE)[192]. SOCE activates Ca^{2+}–calmodulin and its target enzymes and transcription factors, most notably nuclear factor for activated T cells (NFAT) isoforms[193]. Other PM channels are involved in mediating Ca^{2+} signals during T cell activation and include non-selective transient receptor potential (TRP) channels, purinergic ionotropic receptors (P2RX) and CaV channels. Ca^{2+} release by IP_3R (and Ca^{2+} entry through PM channels) is transferred into mitochondria through the mitochondrial Ca^{2+} uniporter (MCU) at highly specialized membrane contact sites termed mitochondria-associated membranes (MAMs), which effectively couple TCR ligation to enhanced bioenergetics and ATP production required for clonal expansion and secretion of cytokines. Sources of Ca^{2+} uptake into endolysosomes remain incompletely understood but contributions from the ER and PM are likely. Endolysosomal Ca^{2+} release controls vesicular fusion, trafficking and secretion of cargo and replenishment of exhausted signalling molecules at the PM of activated T cells[169]. Cytoplasmic, ER and mitochondrial Ca^{2+} homeostasis are maintained by the actions of transporters and pumps, including the PM Ca^{2+} ATPase (PMCA), the sarcoplasmic/ER Ca^{2+} ATPase (SERCA) and the mitochondrial $Na^+/Ca^{2+}/Li^+$ exchanger (NCLX). LAT, linker for activation of T cells; ZAP70, ζ-chain-associated protein kinase of 70 kDa.

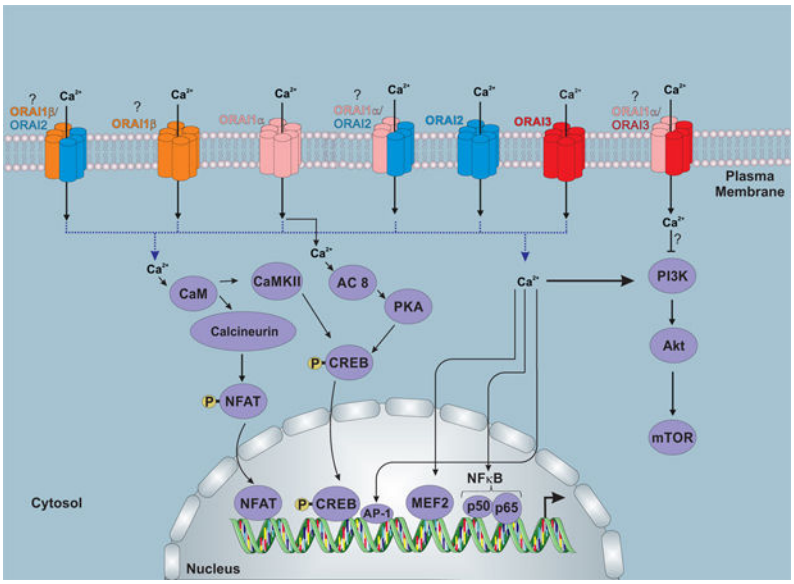

Figure 2 |. ORAI channels: major players in T cell activation.

ORAI proteins form highly Ca^{2+} selective homo-hexameric and hetero-hexameric channels in the plasma membrane (PM). On T cell receptor (TCR) ligation and subsequent inositol-1,4,5-trisphosphate (IP_3)-mediated endoplasmic reticulum (ER) Ca^{2+} store depletion, stromal interaction molecule (STIM) proteins move to ER–PM junctions and physically interact with ORAI channels, causing their activation. Mammals express three ORAI proteins (ORAI1–ORAI3) encoded by three separate genes and the major isoform mediating store operated Ca^{2+} entry (SOCE) in most cells including T cells is ORAI1. ORAI1 exists in two isoforms ORAI1α (long) and ORAI1β (short) due to alternative translation-initiation of Orai1 mRNA[81,194]. ORAI1α and ORAI1β do not seem to form hetero-hexamers. ORAI1β has not been studied in T cells. ORAI2 can mediate residual SOCE in T cells from $Orai1^{-/-}$ mice and was proposed to negatively modulate ORAI1 activity through formation of ORAI1–ORAI2 heteromeric associations. ORAI3, which unlike ORAI1, is resistant to inhibitory oxidation and is upregulated in effector T cells. The exclusively mammalian ORAI1α and ORAI3 proteins were shown to form heteromeric channels that are activated independently of store depletion in other cell types[195], but their existence in T cells is unknown. Ca^{2+} entry through ORAI channels activates calcineurin and subsequent nuclear translocation of isoforms of nuclear factor for activated T cells (NFAT)[193]. ORAI1α physically associates through its N-terminus with the Ca^{2+}-activated adenylyl cyclase 8 (AC8)[196], thus connecting Ca^{2+} microdomains through ORAI1α to cAMP production, activation of protein kinase A (PKA) and the transcription factor cAMP-responsive-element-binding protein (CREB). Ca^{2+} entry through heteromeric ORAI1α–ORAI3 channels was proposed to regulate the AKT pathway[197], although the exact mechanisms remain unknown. AP-1, activator protein-1; CaM, calmodulin; CaMK-II, Ca^{2+}-calmodulin-dependent kinase II; MEF2, myocyte-specific enhancer factor 2; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; PI3K, phosphoinositide 3-kinase.

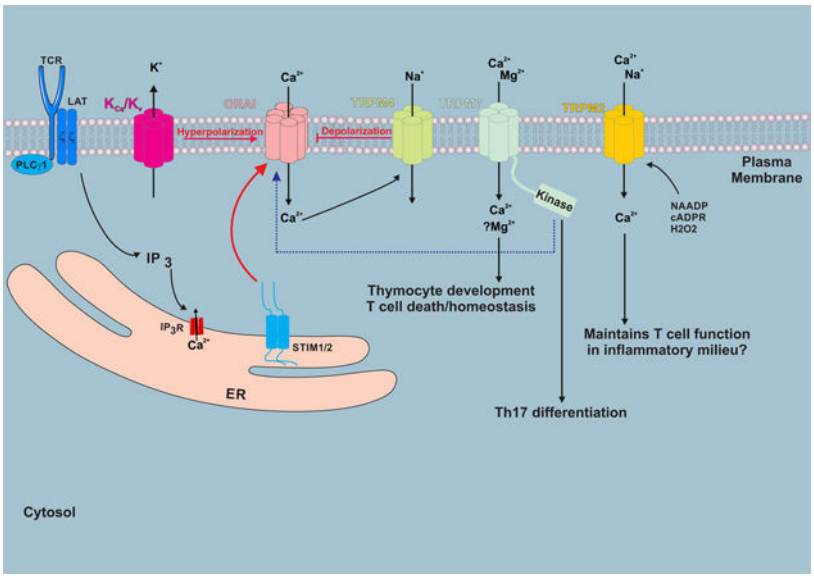

Figure 3 |. TRP channels: regulators of T cell Ca^{2+} signalling.

The transient receptor potential melastatin 4 (TRPM4) is a Ca^{2+}-activated Na+ selective channel that is a potent regulator of ORAI-mediated Ca^{2+} entry in T cells. TRPM4 mediates its action through Na+ entry, causing plasma membrane depolarization, which limits the driving force for Ca^{2+} and inhibits Ca^{2+} entry through ORAI channels. As such, during Ca^{2+} responses in T cells, Ca^{2+} activation by TRPM4 can shape the magnitude of sustained Ca^{2+} signals (plateaus) as well as the frequency and amplitude of oscillatory Ca^{2+} responses and therefore determine which downstream transcription factors and gene programmes are activated. An opposing role is played by Ca^{2+}- and voltage-dependent K+ channels (KCa and Kv), which maintain hyperpolarized Vm to support Ca^{2+} entry. The Mg^{2+}- and Ca^{2+}-permeable channel-enzyme TRPM7 is one of few reported Ca^{2+} channels with a crucial role in T cell development and homeostasis and this function appears to be mediated by Ca^{2+} entry through the channel domain. The kinase domain of TRPM7 regulates ORAI1 signalling and coordinates antigen receptor signalling termination in lymphocytes, likely through phosphorylation of phospholipase Cγ (PLCγ) isoforms[198]. TRPM2 channels are non-selective Ca^{2+}-conducting channels which are activated by hydrogen peroxide (H2O2) and through the cytosolic second messengers nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP ribose (cADPR). TRPM2 has a more established role in neutrophils and was proposed to support T cell Ca^{2+} signalling in high oxidant inflammatory milieu.

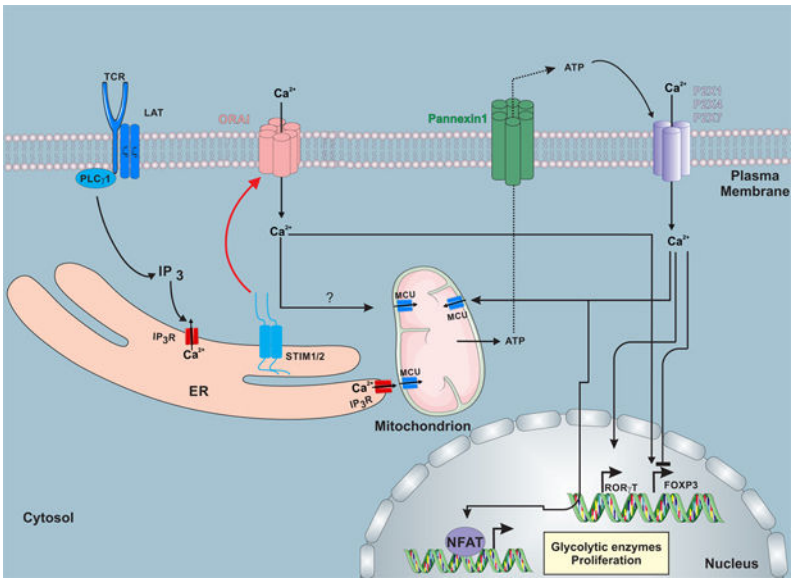

Figure 4 |. P2RX receptors: amplifiers of T cell receptor-mediated Ca^{2+} signalling through paracrine and autocrine ATP.

Purinergic ionotropic receptors (P2RX), including P2RX1, P2RX4 and P2RX7 are trimeric ionotropic non-selective Ca^{2+}-conducting channels that are activated by direct binding of extracellular ATP. Mitochondrial ATP production is enhanced in activated T cells by Ca^{2+} transfer from ORAI- and IP_3R-generated Ca^{2+} microdomains to the mitochondrial matrix via the mitochondrial Ca^{2+} uniporter (MCU)[199]. ATP is then exported outside T cells by the pannexin 1 hemichannels and activates P2RX receptors to cause further Ca^{2+} entry and mitochondrial ATP production. In this regard, P2RX receptor signalling acts as a Ca^{2+} signalling enhancer for nuclear factor for activated T cells (NFAT)-mediated transcription. P2RX7 was shown to promote T helper 17 (TH17) cell differentiation and inflammation by promoting retinoic acid receptor-related orphan receptor γt (RORγt) while inhibiting forkhead box P3 (FOXP3) transcription to suppress regulatory T (Treg) cells. However, Ca^{2+} signals through ORAI1 are required for Treg cell development (for both thymic Treg cells and induced Treg cells in peripheral lymphoid organs).

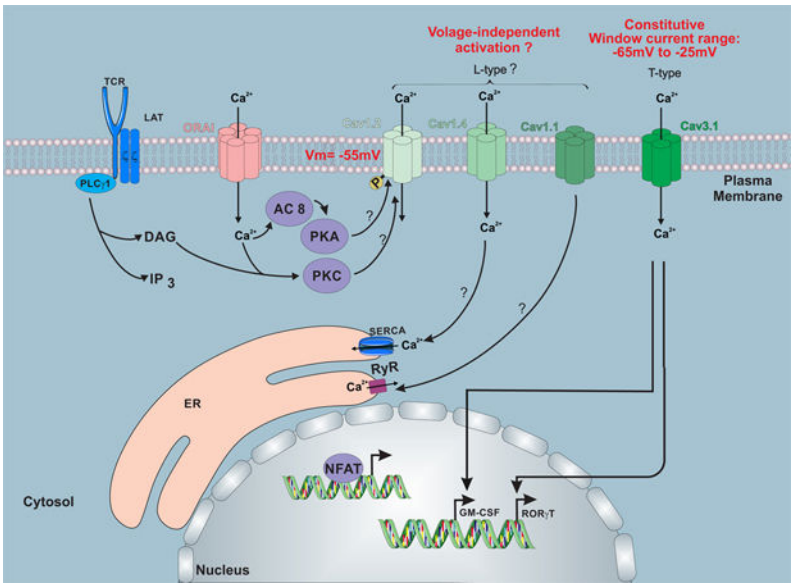

Figure 5 |. CaV channels: modulators of T cell Ca^{2+} signalling.

Based on knockout of the β3 and β4 regulatory subunits of L-type Ca^{2+} channels which causes decreased expression of CaV1.2 and CaV1.1 at the plasma membrane and decreased T cell receptor (TCR)-activated Ca^{2+} entry and nuclear factor for activated T cells (NFAT) activity, CaV1.2 and CaV1.1 were proposed to play a role in TCR-mediated Ca^{2+} signalling. Although no CaV1.2 currents were reported in T cells, there is evidence of spontaneous CaV1.2 currents from channel clusters at hyperpolarized membrane potentials, as low as –90 mV, in smooth muscle cells[135,200]. In this case, CaV1.2 activity can be sensitized by protein kinase A (PKA) and protein kinase C (PKC)-mediated phosphorylation; both enzymes can be activated downstream of store operated Ca^{2+} entry (SOCE). One intriguing hypothesis in T cells is the existence of a conformational coupling between CaV1.1 and ryanodine receptors (RYRs), similar to skeletal muscle. Voltage-activated CaV1.4 was proposed to support SOCE by mediating constitutive Ca^{2+} activity to help refill the ER Ca^{2+} stores and sustain CRAC channel activity, but these studies remain highly controversial. Voltage-activated whole-cell T-type Ca^{2+} currents mediated by CaV3.1 were recorded in T cells, generating window currents between –65 and –25mV, which are within T cell resting membrane potential. CaV3.1-mediated Ca^{2+} entry operates independently of SOCE and provides Ca^{2+} microdomains that synergize with SOCE to support NFAT activity. CaV3.1 activity is required for the expression of GM-CSF and RORγt.

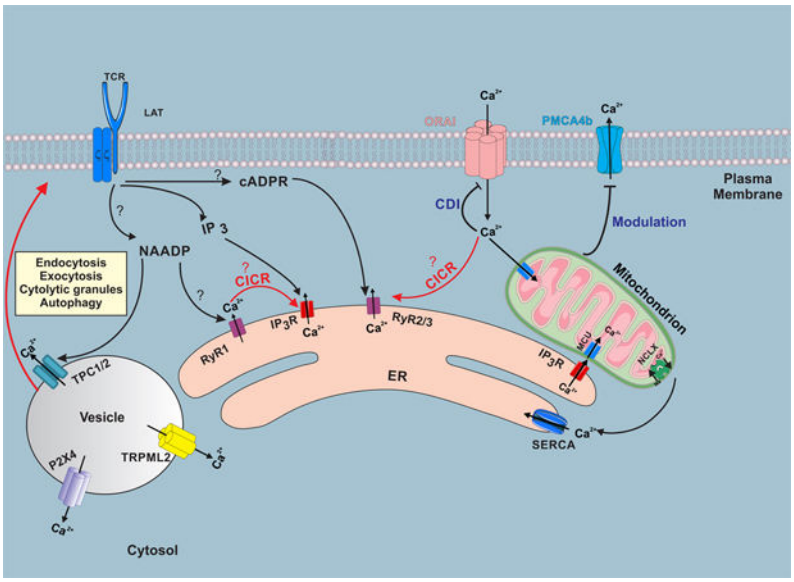

Figure 6 |. Organellar Ca^{2+} channels: initiators and master orchestrators of Ca^{2+} signalling microdomains during T cell activation.

The role of inositol-1,4,5-trisphosphate (IP_3) in initiating and sustaining T cell Ca^{2+} signalling is well established. Nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP ribose (cADPR) were proposed to synergize with IP_3 during T cell Ca^{2+} signalling, but their role requires further clarification. For instance, the identity of the NAADP-producing enzyme downstream TCR ligation and the accessory molecule required for NAADP action on type 1 ryanodine receptors (RYR1) remain obscure. Nevertheless, according to this model, NAADP produced during the first few seconds of T cell receptor (TCR) stimulation was proposed to represent the crucial initial trigger for T cell Ca^{2+} signalling, preceding IP_3-mediated ER Ca^{2+} release. NAADP would activate RYR1 and cause endoplasmic reticulum (ER) Ca^{2+} release, which would synergize with IP_3 to cause further Ca^{2+} release via IP_3 receptor (IP_3R). Subsequent production of cADPR and activation of RYR2 and RYR3 by cADPR would serve to sustain Ca^{2+} signalling for extended periods of time[201]. Through a positive feedback loop, Ca^{2+} entry through ORAI channels might activate RYR isoforms to maintain store depletion and store operated Ca^{2+} entry (SOCE). Mitochondrial Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter (MCU) at the vicinity of ORAI channels maintains CRAC channel activity by relieving its Ca^{2+}-dependent inhibition (CDI). Mitochondria can also buffer Ca^{2+} near plasma membrane Ca^{2+} ATPase (PMCA) pumps, thus preventing PMCA modulation, inhibiting Ca^{2+} extrusion to the outside and maintaining cytosolic Ca^{2+} signalling during T cell activation. Ca^{2+} shuttling to the outside of mitochondria by the activity of the Na+/Ca^{2+}/Li+ exchanger (NCLX) provides Ca^{2+} microdomains to the sarcoplasmic/endoplasmic Ca^{2+} ATPase (SERCA) pump and serves to replenish ER Ca^{2+} levels by these mobile organelles, thus sustaining SOCE and lymphocyte activation. NAADP-mediated activation of lysosomal two pore channels (TPC) is important for secretion of cytolytic granules by cytotoxic T cells. The endolysosomal TRP mucolipin (TRPML) channels are crucial to endolysosomal function, such as endocytosis, exocytosis and autophagy, and are likely important, during T cell activation, for endocytosis of exhausted membrane proteins and replenishment of these

proteins through exocytosis at the immune synapse. Purinergic ionotropic receptor P2RX4 located in endolysosomal membranes was proposed to be important for vesicular fusion[202], but its role in T cell endolysosomal function remains unknown. CICR, Ca^{2+}-induced Ca^{2+} release.