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Immunodeficiency due to defects in store-operated calcium entry

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Abstract

Mutations in genes encoding the Calcium-Release Activated Calcium (CRAC) channel abolish calcium influx in cells of the immune system and cause severe congenital immunodeficiency. Patients with autosomal recessive mutations in the CRAC channel gene *ORAI1*, its activator *Stromal Interaction Molecule 1 (STIM1)* and mice with targeted deletion of *Orai1*, *Stim1* and *Stim2* genes reveal important roles for CRAC channels in adaptive and innate immune responses to infection and in autoimmunity. Since CRAC channels have important functions outside the immune system, ORAI1 and STIM1 deficiency are associated with a unique clinical phenotype. This review will give an overview of CRAC channel function in the immune system, examine the consequences of CRAC channel deficiency for immunity in human patients and mice and discuss genetic defects in immunoreceptor-associated signaling molecules that compromise calcium influx and cause immunodeficiency.

Keywords

Immunodeficiency; T cells; CRAC channels; ORAI1; STIM1

1. CRAC channels are essential for Ca^{2+} influx in cells of the immune system

Ca^{2+} was recognized as a T cell mitogen in the late 1960s¹⁻³ and has since been studied in more detail after the advent of fluorescent Ca^{2+} indicator dyes that enable measurements of intracellular Ca^{2+} levels in single cells. In resting lymphocytes, intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) are kept very low (~100 nM) compared to the much higher concentrations in the extracellular fluid and serum (~1 - 2 mM) and intracellular Ca^{2+} storage compartments such as the endoplasmic reticulum (ER, ~0.5 - 1 mM)⁴. Sustained or oscillatory increases in $[\text{Ca}^{2+}]_i$ may originate in Ca^{2+} influx from the extracellular space via plasma membrane Ca^{2+} channels or the release of Ca^{2+} from intracellular stores. The subsequent increase in $[\text{Ca}^{2+}]_i$ leads to the activation of a multitude of Ca^{2+} dependent proteins, enzymes and transcription factors which regulate the function and, in some instances, development of immune cells. Elevations of $[\text{Ca}^{2+}]_i$ occur following engagement of receptors on adaptive and innate immune cells including T and B cells, NK cells, mast cells, dendritic cells, neutrophils and macrophages⁵. Immunoreceptors that generate Ca^{2+} signals include T cell, B cell, Fc γ R and Fc ϵ R receptors, activating NK cell receptors as well as G-protein coupled chemokine receptors⁵. In T and B cells, antigen binding activates the protein tyrosine kinases lck, syk and ZAP-70 resulting in the phosphorylation of scaffold

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proteins such as LAT, SLP-76, BLNK and the recruitment of the Tec kinases Itk and Btk. Ultimately, these proximal signaling events result in the activation of phospholipase C 1 (in T cells) or PLC 2 (in B cells) and the generation of inositol 1,4,5-triphosphate (InsP₃). The latter binds to InsP₃ receptors in the membrane of the endoplasmic reticulum (ER) triggering the opening of these Ca²⁺ permeable ion channels and the release of Ca²⁺ from the ER. The release of Ca²⁺ from the ER results in a transient and – at least in lymphocytes – very small increase in intracellular [Ca²⁺]_i, which may nevertheless be sufficient for some T cell responses. More importantly, however, the reduction in [Ca²⁺]_{ER} is the trigger mechanism for the induction of store-operated Ca²⁺ entry (SOCE), which is the predominant Ca²⁺ influx pathway in T cells and many other immune cells in response to antigen receptor engagement.

SOCE is mediated by the opening of a Ca²⁺ selective ion channel in the plasma membrane, the Ca²⁺ release activated Ca²⁺ (CRAC) channel. Ca²⁺ currents resulting from the opening of the CRAC channel were first identified in T cells and mast cells^{6, 7} and have since been described in several other immune cells as well as cells outside the immune system^{5, 8, 9}. The opening of CRAC channels results in sustained Ca²⁺ influx and elevations in [Ca²⁺]_i that are required for efficient stimulation of immune cells. The importance of CRAC channels for immunity to infection is emphasized by rare but very instructive patients with inherited defects in SOCE due to mutations in genes of the CRAC channel complex. These patients suffer from a severe form of immunodeficiency that is due to defects in the function, but not the development, of T cells, NK cells and potentially other immune cells^{10, 11} as will be discussed in detail below.

2. CRAC channel function is controlled by ORAI and STIM proteins

The genes encoding the CRAC channel remained elusive until 2006 when a genomic linkage analysis in patients with inherited CRAC channel deficiency and functional RNAi screens in *Drosophila* S2 cells resulted in the identification of *ORAI1* as the gene encoding the CRAC channel located on human Chr 12q24¹²⁻¹⁴. *ORAI1* (also known as CRACM1), and its two homologues *ORAI2* and *ORAI3*, represent a new class of Ca²⁺ channels that are unrelated to other ion channels. They are named after the Horae (hours) Eunomia, Dyke and Eirene in Homer's *Iliad*¹⁵. All three ORAI proteins are tetraspanning plasma membrane channels with intracellular N- and C-termini and two extracellular loop regions. The first transmembrane domain (TM1) of *ORAI1* contains an important negatively charged glutamate residue (E106) that was shown to function as a Ca²⁺ binding site inside the CRAC channel pore¹⁶⁻¹⁸. TM1 was later confirmed to be an alpha-helix lining the channel pore and essential for conducting Ca²⁺¹⁹⁻²¹. In their active state, CRAC channels are thought to be composed of four *ORAI1* proteins²²⁻²⁴. *ORAI2* and *ORAI3* are calcium channels with electrophysiological properties that are largely similar to those of *ORAI1* when they are overexpressed in cell lines^{25, 26}, but the role of endogenous *ORAI2* and *ORAI3* proteins for Ca²⁺ influx is not well understood.

CRAC channels are activated by the depletion of ER Ca²⁺ stores. The mechanisms underlying this activation remained as controversial as the nature of the CRAC channel itself. In 2005, stromal interaction molecule (STIM) 1 was identified as protein essential for CRAC channel activation^{27, 28}. STIM1, and its homologue STIM2, are single-pass transmembrane proteins that are localized in the ER²⁹. Their ER luminal N terminus contains a pair of low affinity EF hand Ca²⁺ binding domains that sense the filling state of the ER. Mutation of acidic glutamate or aspartate residues within the EF hand results in constitutive CRAC channel activation and SOCE³⁰. The cytoplasmic C terminus of STIM1 contains two coiled-coil domains which mediate protein interactions between STIM1 molecules to form multimeric STIM1 complexes²⁹. The more C terminal coiled-coil

domain of STIM1 is part of functional protein domain that mediates the binding of STIM1 to ORAI1 resulting in CRAC channel opening. This domain is alternatively called CAD (CRAC activating domain)³¹, SOAR (STIM-ORAI-activating region)³², OASF (ORAI1-activating small fragment)³³ and CCb9 (coiled-coil domain fragment b9)³⁴. Several other domains in the C terminus of STIM1 have also been shown to regulate SOCE and the function of other ion channels such as transient receptor potential (TRP) C1 and L-type voltage-gated Ca^{2+} channels³⁵⁻³⁸. Similar to STIM1, STIM2 senses $[\text{Ca}^{2+}]_{\text{ER}}$ and activates SOCE, albeit with different thresholds of activation and kinetics^{39, 40}. It has therefore been suggested that STIM2 mainly regulates basal cytosolic Ca^{2+} concentrations⁴¹.

ORAI1 and STIM1 are widely expressed in the immune system and other tissues^{10, 42-45}. In mice, similar levels of ORAI1 protein are found in all lymphocyte populations⁴⁶. Above-average mRNA levels are, however, observed in macrophages and mast cells⁴². ORAI1 is in addition expressed at high levels in skeletal muscle and many secretory epithelia of exocrine organs such as the mammary, salivary, pancreatic and prostate glands^{10, 43}. It is of note that the levels of ORAI1 and STIM1 in cells of the immune system are not or only moderately higher compared to those in other tissues⁴⁴. The fact that mutations in *ORAI1* and *STIM1* genes in human patients mainly manifest as immunodeficiency, as will be discussed further below, is presumably due to the non-redundant function of both proteins in Ca^{2+} influx in cells of the immune system compared to other tissues.

3. The role of SOCE in immune cells

Ca^{2+} signals in cells of the adaptive and innate immune system have been associated with numerous cell functions. SOCE is the main Ca^{2+} influx pathway in lymphocytes and other immune cells following antigen stimulation via TCR, BCR or Fc receptors. Other Ca^{2+} permeable channels and pathways have, however, been described as well such as P2X receptors in mast cells and T cells⁴⁷⁻⁵⁰, TRPC channels and plasma-membrane expressed InsP_3 receptors in B cells⁵¹⁻⁵³. The contribution of these channels to immune responses *in vivo* remains largely unclear.

Since the discovery of *ORAI* and *STIM* genes, research on Ca^{2+} influx in immune cells has focused on the role of SOCE mediated by CRAC channels. A detailed review of how SOCE regulates the function of T cells, B cells, NK cells, mast cells, neutrophils and macrophages can be found in⁵⁴. Briefly, one of the most important roles of SOCE in all immune cells is the regulation of gene expression via the activation of Ca^{2+} dependent transcription factors such as the nuclear factor of activated T cells (NFAT)⁵⁵, cAMP response element binding (CREB)⁵⁶, myeloid elf-1-like factor (MEF) 2⁵⁷ or activating transcription factor (ATF) 2⁵⁸. In particular, the production of many cytokines and chemokines is regulated by SOCE⁵⁹⁻⁶¹. In addition, SOCE is required for the release of cytolytic granules by CD8^+ T cells and NK cells following stimulation through the TCR and Fc RIIIa/b (CD16), respectively^{61, 62}. In mast cells, SOCE mediated by ORAI1 and STIM1 is essential for the production of leukotriene C4 as well as the release of histamine and serotonin containing granules in response to Fc RI crosslinking^{63, 64}.

CRAC currents and SOCE have also been reported in other myeloid cells. In dendritic cells (DC), CRAC currents were recorded after store-depletion with thapsigargin or ATP stimulation⁶⁵ and have been implicated in DC maturation as measured by cell surface expression of MHC class II, costimulatory molecules and chemokine receptors^{66, 67}. More recently it was shown that in neutrophils, the production of reactive oxygen species and cell motility were dependent on ORAI1 and STIM1 expression⁶⁸⁻⁷⁰. Although these experiments were conducted mostly in the HL-60 neutrophil cell line, the findings are in line with older studies that demonstrated Ca^{2+} influx following crosslinking of Fc RIIIa and

Fc γ IIIb receptors, binding of chemokine receptors by IL-8 or fMLP⁷¹⁻⁷³ and a even older work linking Ca^{2+} influx to the production of reactive oxygen species (ROS)⁷⁴. More direct evidence for a role of SOCE in innate immune responses comes from experiments in STIM1 deficient mice, whose macrophages failed to phagocytose opsonized red blood cells *in vitro* and to mediate Fc γ R dependent autoimmune hemolytic anemia and thrombocytopenia *in vivo*⁷⁵. Taken together, it is now appreciated that SOCE through CRAC channels constitutes a universal Ca^{2+} signaling pathway in cells of the immune system and likely to be involved in both adaptive and innate immune responses. The precise contributions of SOCE in different cell types to immunity *in vivo* still remain to be investigated.

4. CRAC channelopathy due to mutations in ORAI1 and STIM1

Even before the discovery of *ORAI1* and *STIM1* genes as the principal components of the CRAC channel complex, patients from three independent families with defects in SOCE and CRAC channel function had been described, who suffered from a Combined Immunodeficiency (CID) syndrome with severe infections to which the patients succumbed in their first year of life^{10, 76-78}. All three patients later emerged to have mutations in *ORAI1*. *ORAI1* was identified as the CRAC channel gene in part through positional cloning using DNA from one of these patient's families¹². The clinical and cellular phenotypes of the patients will be discussed further below.

Mutations in *ORAI1*

Autosomal recessive mutations in *ORAI1* have been identified in three unrelated kindreds so far (**Figure 2, Table 1**): 1. A missense mutation in the pore-lining first transmembrane (TM) domain of *ORAI1* (R91W) results in the expression of a non-functional CRAC channel¹² and abolishes SOCE in T cells, B cells, NK cells and fibroblasts of patients^{12, 61, 79}. R91 is located at the inner mouth of the CRAC channel pore and substitution of the basic Arg residue with a hydrophobic Trp was shown *in silico* to enhance the transmembrane probability of TM1, thus potentially interfering with the structure or mobility of TM1 in the lipid bilayer and the opening of the CRAC channel pore⁸⁰. 2. Missense mutations in the first and third TM domains of *ORAI1* (A103E, L194P) abolish protein expression, presumably by destabilizing the alpha-helical structure of the membrane domains^{10, 77}. Immunodeficient patients are compound heterozygous for both mutations. 3. An insertion mutation (A88SfsX25) results in a frameshift, nonsense mediated mRNA decay and lack of *ORAI1* protein expression^{10, 78}. These mutations have been described in detail elsewhere^{10, 43, 46, 81}.

Only individuals homozygous or compound heterozygous for the mutations described above present with immunodeficiency and the non-immunological symptoms of CRAC channelopathy (**Figure 3**), whereas heterozygous individuals (parents, siblings) are healthy. Their T cells (and other cell types tested) have normal SOCE at physiological extracellular Ca^{2+} concentrations indicating that monoallelic expression of *ORAI1* is sufficient for SOCE, T cell function and immunity to infection. The same is true for patients with mutations in *STIM1* that will be discussed below. It is noteworthy, however, that heterozygosity for the *ORAI1*-R91W mutations reduces CRAC channel currents by ~ 50%⁸². SOCE is also reduced by ~ 50% but only at subphysiological (< 1.2 mM) extracellular Ca^{2+} concentrations^{12, 82}. This is in contrast to cells from individuals heterozygous for null mutations (A103E, L194P, A88SfsX25) which have normal SOCE¹⁰, suggesting that the R91W mutation has a dominant negative effect on CRAC channel function. This is plausible as the mutant protein subunits are likely to be assembled into the tetrameric *ORAI1* channel complex²²⁻²⁴. Experiments using concatenated tetramers of wildtype and mutant (R91W) *ORAI1* proteins confirmed this dominant negative effect⁸³. One mutant subunit was sufficient to reduce CRAC currents by ~ 50%, whereas two

completely abolished channel function. The less severe effect of heterozygously expressed ORAI1-R91W proteins on CRAC channel function may be explained by a decreased incorporation of mutant subunits into the tetrameric ORAI1 complex in heterozygous individuals unlike in concatenated tetramers, where the ratio of wildtype to mutant subunits is fixed at 50%.

Mutations in STIM1

Inherited mutations in STIM1 that impair SOCE and cause immunodeficiency are equally rare as those in ORAI1. To date, four patients from two families have been reported, who are homozygous for autosomal recessive mutations in *STIM1* that abolish mRNA and protein expression (**Figure 2, Table 1**)^{11, 84}. Patients from a third family have recently been identified which lack SOCE due to a missense mutation in STIM1, but the molecular mechanism that causes the defect in Ca²⁺ influx in cells from these patients has not been resolved yet⁶¹. Three patients from one family were homozygous for an adenine insertion in exon 3, which results in a frameshift, premature stop codon and termination at position 136 of the protein sequence (E128RfsX9). A predicted 15 kDa N-terminal STIM1 fragment, however, was not observed. Instead, STIM1 mRNA levels were greatly reduced and STIM1 protein expression was not detectable in the patients' cells suggestive of nonsense mediated mRNA decay¹¹. STIM1 protein expression was also abolished in a patient from another family whose B cells lacked SOCE⁸⁴. She was homozygous for a splice site mutation in exon 8 of STIM1 (1,538-1 G>A) and lacked normally spliced, full-length STIM1 mRNA transcripts. Neither full-length nor truncated STIM1 proteins (representing abnormal STIM1 splice products) were detectable in the patient's B cells. It is noteworthy that expression not only of wildtype STIM1 but also STIM2 was able to rescue SOCE in fibroblasts from one of the STIM1 deficient patients¹¹ suggesting that STIM1 and STIM2 have overlapping functions in CRAC channel activation. Nevertheless, even the above average endogenous mRNA expression levels of STIM2 in human CD4⁺ and CD8⁺ T cells, CD56⁺ NK cells and CD19⁺ B cells compared to other cell types⁴² appear to be insufficient to compensate for a loss of STIM1 expression and prevent immunodeficiency in STIM1 deficient patients.

The complete loss of SOCE in ORAI1 and STIM1 deficient patients and their severe (and to a large degree overlapping) clinical phenotype indicate that STIM1 and ORAI1 are the major protagonists in CRAC channel function and SOCE in human immune cells. By contrast, ORAI2, ORAI3 and STIM2 appear to play only a minor role as their endogenous expression was unable to mediate SOCE and to prevent the patients' immunodeficiency. The physiological role of ORAI2, ORAI3 and STIM2 for CRAC channel function and Ca²⁺ influx in human lymphocytes remains to be resolved. It has been speculated that ORAI2 is important for SOCE in murine T cells⁶⁴, but evidence for such a role in human T cells is missing. Above average mRNA expression levels of ORAI2 and ORAI3 were observed in human CD19⁺ B cells and CD14⁺ monocytes, respectively⁴², suggesting their possible involvement in SOCE in these cells. STIM2 is required for murine T cell function, as T cells from mice with conditional deletion of the *Stim2* gene have a significant defect in sustaining Ca²⁺ influx, which results in impaired cytokine production *in vitro* and partial protection of *Stim2* deficient mice from T-cell mediated autoimmune diseases⁸⁵⁻⁸⁷. The role of STIM2 in SOCE and immunity in humans needs to be investigated. Taken together, existing data from ORAI1 and STIM1 deficient patients and gene-targeted mice indicate that ORAI1 and STIM1 are the predominant CRAC channel proteins in human and murine T cells, B cells, NK cells and potentially other immune cells.

5. Clinical phenotype of CRAC channelopathy

Autosomal recessive mutations in ORAI1 and STIM1 are the cause of a unique disease syndrome we termed CRAC channelopathy. It is characterized by severe immunodeficiency,

autoimmunity, muscular dysplasia and ectodermal dysplasia (**Figure 3, Table 1**). The clinical disease spectrum in *ORAI1* and *STIM1* deficient patients is largely identical, indicating that both genes act – more or less exclusively – in the same signaling pathway. Although the number of patients with mutations in *ORAI1* and *STIM1* genes observed to date is small, *ORAI1* deficient patients appear to have more severe disease since they die in the first year of life without hematopoietic stem cell transplantation (HSCT)¹⁰. By contrast, most *STIM1* deficient patients survived the first year of life without treatment^{11, 84}, which may also explain the higher overall incidence of autoimmunity in these individuals.

5.1. Immunodeficiency

The immunodeficiency in CRAC deficient patients is characterized by recurrent severe infections with viral, bacterial, fungal and, in one case, mycobacterial pathogens (**Table 1**)^{10, 11, 84}. Patients are frequently affected by herpes virus infections including those with cytomegalovirus (CMV), Epstein-Barr Virus (EBV) and varicella zoster virus. Infections with *Streptococcus pneumoniae*, *Escherichia coli* and other bacteria have caused otitis media, pneumonia, gastroenteritis, urinary tract infections, meningitis and sepsis. In addition, CRAC deficient patients suffered from infections with *Toxoplasma gondii*, *Candida albicans* and *Bacille-Calmette Guérin* (BCG), an attenuated vaccination strain of *Mycobacterium bovis*⁷⁶. While most patients suffered from repeated infections with multiple classes of pathogens, one *STIM1* deficient patient was unusual, as she did not have recurrent severe infections. She did, however, develop disseminated Kaposi sarcoma (KS) two years of age due to infection with human herpes virus 8 (HHV8)^{84, 88}. She died from severe pulmonary infection three months after the beginning of antitumor therapy. The severity of infections, their time of onset in the first year of life and the spectrum of pathogens in CRAC deficient patients is similar to those with Severe Combined Immunodeficiency (SCID) who have a impaired T and/or B cell development and immunity. In contrast to SCID patients, T and B cell development is normal in CRAC deficient patients. Their immunodeficiency is due to the impaired function of T cells^{11, 59, 76, 77}, NK cells⁶¹ and most likely other cells of the innate immune system.

The immunodeficiency in CRAC deficient patients is severe because of the 10 patients with confirmed or strongly suspected mutations in *ORAI1* and *STIM1* genes, only three (one patient with *ORAI-R91W*, *ORAI1-A103E/L194P* and *STIM1-E128RfsX9* mutation each)⁸¹, have survived after HSCT, whereas five succumbed to infections < 2 years of age and two died from treatment complications. Like in SCID, infections in *ORAI1* and *STIM1* deficient patients respond poorly to treatment with antibiotics or intravenous immunoglobulin (IVIg) substitution and HSCT is the only curative therapy.

5.2. Autoimmunity

Lymphoproliferation and autoimmune cytopenias were observed in all *STIM1* deficient patients known to date^{11, 84}. Besides lymphadenopathy and hepatosplenomegaly, the patients suffered from Coombs-positive hemolytic anemia and thrombocytopenia associated with autoantibodies against platelet glycoproteins Ib/IX¹¹. The most likely cause of the patients' autoimmunity is the reduced number of CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells (Treg) that was observed in the blood of a *STIM1* deficient patient¹¹. Treg cells are essential for maintaining immunological tolerance to self-antigens and their development was severely impaired in mice with T-cell specific deletion of *Stim1* and *Stim2* genes⁸⁶. In these mice, the numbers of Treg cells in the thymus and secondary lymphoid organs were reduced to ~ 10% of the levels found in wildtype controls. In addition, the suppressive function of the residual *Stim1/Stim2* deficient Treg cells was severely impaired. These mice develop a phenotype similar to those of *STIM1* deficient patients which includes lymphadenopathy, splenomegaly, autoantibodies and inflammatory infiltration of the lung with eosinophils,

basophils and lymphocytes, colitis, dermatitis and blepharitis⁸⁶. By contrast, mice with T-cell specific deletion of *Stim1* alone or knockin mice homozygous for the non-functional *Orai1-R93W* mutation have normal Treg numbers and partially impaired Treg function in vitro^{82, 86, 89}.

An alternative explanation for the autoimmunity in *STIM1* deficient patients and *Stim1/Stim2* deficient mice is an aberrant selection of autoreactive T cells and/or B cell during their development in the thymus and bone marrow due to altered TCR signaling thresholds in the absence of Ca²⁺ influx. The T cell receptor repertoire (analyzed using monoclonal antibodies against the TCR α chain variable regions V 3, V 8, V 13-6, V 13-17, and V 21-3) in a patient lacking *STIM1* expression, however, was normal¹¹, and similar observations were made in *Stim1/Stim2* deficient mice.

In contrast to *STIM1* deficient patients, autoimmunity and lymphoproliferation have so far only been observed in one out of six *ORAI1* deficient patients who presented with autoimmune thrombocytopenia and neutropenia¹⁰. The cause of the discrepancy between *ORAI1* and *STIM1* deficient patients is not known, partially owing to the fact that blood samples from *ORAI1* deficient patients to analyze the number of Foxp3⁺ Treg cells were not available. A simple explanation for the lower incidence of autoimmunity in *ORAI1* compared to *STIM1* deficient patients may be their early mortality. Analysis of a larger patient cohort will be necessary to determine whether *STIM1* may have a gene-specific role in the development of autoimmunity.

Despite the reduced numbers of Treg cells, the autoimmunity in *STIM1* deficient patients and *Stim1/Stim2* deficient mice is less pronounced than that observed in patients with IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome or scurfy mice which lack Treg cells due to mutations in the human and mouse genes encoding FOXP3. This is most likely due to the fact that FOXP3⁺ Treg cells are not completely absent in *STIM1* deficient compared to IPEX patients⁹⁰. In addition, effector functions of potentially autoreactive T and B cells are also impaired in the absence of *STIM1* and *SOCE*, thereby attenuating the severity of autoimmune disease. *Stim1* deficient (and to a lesser degree also *Stim2* deficient) mice were protected from developing autoimmune CNS inflammation in a murine model of multiple sclerosis^{85, 87} and autoimmune colitis⁸².

5.3. Non-immunological symptoms

CRAC channelopathy is associated with a unique combination of immunological and nonimmunological symptoms that is pathognomonic and facilitates the diagnosis of this PID. The non-immunological symptoms include congenital, generalized muscular hypotonia, a dental enamel calcification defect (amelogenesis imperfecta type III) and an inability to sweat (anhidrosis) that is presumably due to impaired sweat gland function^{10, 11, 81}. These defects have been discussed in detail elsewhere and will only briefly be discussed here^{43, 46}. The muscular dysplasia in *ORAI1* deficient patients is characterized histologically by an atrophy of type II muscle fibers¹⁰. It constitutes a severe clinical problem in patients who survived after HSCT and are now 9-17 years old^{10, 11, 61}. Besides resulting in the partial immobilization of *ORAI1* deficient patients, a muscular hypotonia of the respiratory musculature impairs expectoration, mucus clearance and resulted in the development of chronic pulmonary disease. A similar nonprogressive muscular hypotonia as well as partial iris hypoplasia were observed in *STIM1* deficient patients, although the muscle biopsy and electromyography did not reveal abnormalities^{11, 81}.

The muscular dysplasia in CRAC channel deficient patients is consistent with an important role of *SOCE* in the function and potentially the development of skeletal muscle^{43, 91, 92}. This notion is supported by the defective function of skeletal muscle fibers from *Stim1*^{-/-}

mice, which fatigued rapidly upon repeated stimulation and showed a decrease in tetanic force⁹³. Skeletal muscle isolated from *Stim1* deficient mice also showed a reduction in muscle fiber diameter and a mitochondriopathy⁹³. By contrast, skeletal muscle from *Orai1* knockin mice (in which the wildtype *Orai1* gene was replaced with a mutated version that encodes for the non-functional ORAI1-R93W protein that is analogous to ORAI1-R91W in human patients) did not show structural abnormalities⁸². Skeletal muscle function was not tested in these mice. The reason for the discrepancy between *Stim1* and *Orai1* deficient mice is not clear. Taken together, CRAC channels emerge to have important roles in the refilling of SR calcium stores, contraction and potentially also the differentiation of skeletal muscle fibers.

6. Cellular defects underlying immune dysregulation in CRAC deficiency

The immunodeficiency in CRAC channel deficient patients and mice is due to impaired activation of T cells^{59, 76, 77, 82}, NK cells⁶¹ and potentially other innate immune cells (see section 3 above).

The immunodeficiency in ORAI1 and STIM1 deficient patients most closely resembles that observed in CID patients with defects in T-cell mediated immunity. In contrast to SCID patients, T cell development (and that of most other lymphoid and myeloid lineages for that matter), is normal in the absence of SOCE^{10, 11, 77, 78, 82} with the notable exception of regulatory T cells⁸⁶. The numbers of CD4⁺ and CD8⁺ T cells, CD16⁺ CD56⁺ NK cells and CD19⁺ or CD20⁺ B cells in SOCE deficient patients were largely normal^{10, 11}. Normal development of T, B and NK cells has also been observed in mice lacking *Orai1*, *Stim1*, *Stim2* and *Stim1/Stim2* gene expression^{63, 64, 82, 86, 89}. In addition, the T cell receptor repertoire (as measured by the distribution of V chains) in STIM1 deficient patients was comparable to that in healthy controls¹¹. These findings are surprising, as Ca²⁺ signals had been observed in immature T cells in response to pre-TCR stimulation and had been implicated in the development and positive selection of T cells in the thymus^{94, 95}. In addition, many immunodeficiencies that are due to defects in T or B cell signaling molecules are associated with defects in SOCE and lymphocyte development (see section 7). Since Ca²⁺ influx following TCR and BCR stimulation is predominantly if not exclusively mediated by ORAI1 and STIM1 (see section 4) and neither patients nor mice lacking expression of these genes have defects in T or B cell development^{11, 12, 60, 64, 82, 89}, the role of Ca²⁺ signals in lymphocyte development has to be revisited. It is possible that Ca²⁺ influx following pre-TCR stimulation is not required for T cell development. Alternatively, the release of Ca²⁺ from intracellular Ca²⁺ stores (which is largely intact in T cells lacking ORAI1 or STIM1 expression^{12, 86}) may be sufficient to promote T cell development. Another possibility is that STIM-independent, non-store operated Ca²⁺ channels mediate Ca²⁺ influx in immature T cells.

By contrast, SOCE is essential for T cell function. This notion is supported by the spectrum of pathogens (**Table 1**) that causes infections in SOCE deficient patients (which includes viruses and *C. albicans*, immunity to which depends on CD8⁺ T cells and Th17 cells, respectively), the defects in T cell function observed in SOCE deficient human and mouse T cells^{59, 76, 82, 89} and the important role of ORAI1, STIM1 and STIM2 in T cell mediated immune responses identified using gene-targeted mice^{82, 89}. In human T cells, SOCE deficiency results in a severe defect in T cell proliferation in response to stimulation with anti-CD3 antibodies, recall antigens (tetanus toxoid, PPD, candidin) and mitogens (PHA, ConA and PMA plus ionomycin)⁷⁶. SOCE regulates the expression of hundreds of genes in CD8⁺ T cells including those encoding cytokines, chemokines and cell surface receptors⁵⁹. Prominent among the genes that require SOCE for their expression are the cytokines IL-2, IL4, IL-10, IFN- γ , TNF- α and the chemokines CCL3 (MIP-1 α), CCL4 (MIP-1 β) or

lymphotactin^{59, 76}. A similar defect in cytokine production by T cells was observed in mice with targeted deletion of *Orai1*, *Stim1* or *Stim2*^{64, 82, 86, 89}. This defect transcends the boundaries of helper cell (Th) subsets as the production of IFN γ by Th1 cells, IL-4 and IL-10 by Th2 cells as well as IL-17A, IL-17F and IL-22 by Th17 cells was impaired in *Orai1* and *Stim1* deficient mice^{64, 82, 86, 89}. T-cell mediated immune responses *in vivo* also required SOCE. ORAI1 deficient patients lacked delayed-type hypersensitivity responses following intraepidermal challenge with recall antigens and T-cell dependent antibody production^{76, 77}. *Orai1* knockin mice lacking expression of functional ORAI1 had impaired T-cell mediated immune responses *in vivo*. They failed to mount a T-cell dependent hypersensitivity response following epicutaneous application of a contact allergen and showed impaired rejection of HLA mismatched skin allografts⁸². In addition, mice lacking *Stim1* or *Stim2* selectively in T cells or in all hematopoietic cells were protected from T-cell mediated autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and inflammatory bowel disease^{85, 87}.

In contrast to the defect in T cell activation following TCR stimulation, T cell from patients with mutations in *ORAI1* and *STIM1* have an activated phenotype with increased numbers of HLA-DR⁺, CD45RO⁺ or CD29⁺ T cells^{10, 11}. A similar increase in activated CD44^{hi} CD62L^{lo} T cells (both CD4⁺ and CD8⁺) is observed in T cells from *Orai1*, *Stim1* and especially *Stim1/Stim2* deficient mice^{82, 86}. Such TCR and SOCE independent T cell activation might also contribute to the autoimmune lymphoproliferative phenotype present in ORAI1 and STIM1 deficient patients.

The susceptibility to viral infections observed in ORAI1 and STIM1 deficient patients points to an important role of SOCE in antiviral immune responses by CD8⁺ T cells and NK cells. SOCE in human and mouse CD8⁺ T cells and NK cells is mediated by ORAI1 and STIM1^{59, 61, 82}. Consequently, CD8⁺ T cells and NK cells from ORAI1 deficient patients and mice lacked expression of TNF α and IFN γ ^{61, 82, 96}. In addition, both CD8⁺ T cells and NK cells from ORAI1 and STIM1 deficient patients showed severe defects in cytotoxic granule exocytosis and in cell-mediated cytotoxicity of tumor cells⁶¹. Similar defects were observed in SOCE deficient CD8⁺ T cells and NK cells isolated from *Orai1* knock-in mice⁸². Taken together, these findings suggest that SOCE is critical for cytotoxic function of CD8⁺ T cells and NK cells in antiviral (and potentially also antitumor) immune responses and that the increased susceptibility to infections in SOCE deficient patients is likely to be due to defects in both adaptive and innate immunity.

Despite the reported role of SOCE in B cell activation⁹⁷, severely impaired Ca²⁺ influx in B cells from ORAI1 and STIM1 deficient patients and mice does not seem to result in an overt B cell defect. The development and maturation of B cells was normal in ORAI1 or STIM1 deficient patients^{10, 11} and mice with conditional deletion of *Stim1* and *Stim2* genes in B cells⁶⁰ or knock-in mice expressing a non-functional form of *Orai1*⁸². Likewise, B cells are able to differentiate into Ig producing plasma cells and to produce antibodies in the absence of SOCE. Serum IgG, IgM and IgA levels were essentially normal in ORAI1 and STIM1 deficient patients^{11, 98}. ORAI1 deficient patients did, however, lack antigen-specific antibody responses after vaccination against tetanus, diphtheria and poliovirus^{10, 76, 78}, suggesting that the function of follicular helper T cells may require SOCE⁷⁶. In contrast to ORAI1 deficient patients, *Stim1*^{-/-} mice showed a normal humoral immune response to immunization with keyhole limpet hemocyanin (KLH); serum titers of anti-KLH antibodies before and after booster immunizations were similar in *Stim1* deficient and wildtype mice⁸⁹. Similar observations were made in mice with B-cell specific deletion of both *Stim1* and *Stim2* genes, which showed normal primary and memory antigen-specific antibody responses, normal affinity maturation and had similar numbers of germinal center B cells compared to wildtype mice⁶⁰. Collectively, these data show that – in contrast to humans –

SOCE in murine T cells and B cells is dispensable for antigen-specific antibody responses⁸⁹. The cause for the discrepancy between humans and mice is not known.

7. Other immunodeficiencies associated with defects in SOCE

Mutations in *ORAI1* and *STIM1* genes are not the only inherited gene defects that are associated with impaired SOCE and immunodeficiency. Inborn errors in immunity to infection can also result from mutations in molecules transmitting signals from the T cell or B cell receptor that regulate – among other pathways – the release of Ca^{2+} from ER stores and thus SOCE. Since most of these molecules are specifically expressed in T and B cells, the resulting immunodeficiency is largely limited to these cell types in contrast to the more universal role of SOCE, *ORAI1* and *STIM1* in many cell types within the immune system and beyond. Examples for gene defects that affect SOCE and immune function are mutations in the zeta-chain-associated protein kinase 70 (ZAP-70) in T cells⁹⁹⁻¹⁰¹, the IL-2-inducible T cell kinase (ITK) in T cells¹⁰², Bruton's tyrosine kinase (BTK)¹⁰³⁻¹⁰⁵ in B cells and the B cell linker protein (BLNK, also known as SLP-65) in B cells¹⁰⁶.

Loss-of-function mutations in ZAP-70 cause combined immunodeficiency (CID) and are associated with impaired development of CD8^+ T cells and defective activation of CD4^+ T cells⁹⁹⁻¹⁰¹. Ca^{2+} influx was found to be significantly impaired in ZAP-70 deficient T cells after TCR stimulation but could be rescued – in contrast to patients with mutations in *ORAI1* or *STIM1* – by direct depletion of ER Ca^{2+} stores and thus activation of SOCE with ionomycin¹⁰⁰. Whereas SOCE was abolished in T cells isolated from the peripheral blood of ZAP-70 deficient patients, thymocytes showed only a partial defect in Ca^{2+} influx. The difference has been explained by the higher expression levels of the src kinase Syk in immature versus mature human T cells and its role in compensating for the loss of ZAP-70 function¹⁰⁷.

ITK is a tyrosine kinase of the Tec family that is activated by TCR stimulation and recruited into a complex with the adaptor protein SLP-76, where it mediates the phosphorylation of PLC and production of InsP_3 . Accordingly, SOCE in response to TCR stimulation was significantly reduced in *Itk*^{-/-} mice¹⁰⁸. These mice had reduced numbers of mature thymocytes¹⁰⁹ and CD4^+ T cells in the periphery¹⁰⁸. The only two known *ITK* deficient patients, by contrast, had normal T cell numbers, which were mostly CD45RO^+ memory T cells¹⁰². By contrast, both *ITK* deficient patients and mice lacked NKT cells, potentially explaining the severe immune dysregulation and susceptibility to EBV infection observed in the patients¹⁰². Unfortunately, SOCE has not been measured in the patients' T cells.

In B cells, mutations in the Tec kinase *BTK* and the scaffold protein *BLNK* in human patients are the cause of X-linked and autosomal recessive agammaglobulinemia, respectively¹⁰⁶. BLNK is phosphorylated by the tyrosine kinase Syk following stimulation of the BCR in mature and the pre-BCR in immature B cells. This results in the recruitment of BTK and PLC 2 into a trimolecular complex with BLNK and the phosphorylation of PLC 2 by BTK⁹⁷. Not surprisingly then, Ca^{2+} influx in pre-B cells isolated from the bone marrow of BTK deficient patients was severely impaired following crosslinking of the μ -heavy chain¹¹⁰. Similarly, crosslinking of the BCR on B cells isolated from BLNK deficient mice failed to induce SOCE¹¹¹. Since signaling through the pre-BCR complex is required for survival of immature B cells, B cell development in *BTK* and *BLNK* deficient patients and mice is compromised at the transition of immature to mature B cells^{106, 111}. The ensuing failure to produce antibodies in these patients results in an increased susceptibility to bacterial infections.

Defects in additional signaling molecules in mouse T and B cells have been associated with impaired SOCE and immune dysregulation. The conditional deletion of PLC 1 in mouse T

cells, for instance, abolishes SOCE¹¹², whereas a partial defect in Ca²⁺ influx is observed in bone marrow derived mast cells from *Slp76*^{-/-} and *Lat*^{-/-} mice^{113, 114}. Taken together, defects in many immunoreceptor-associated signaling molecules can affect SOCE in T and B cells, and it is tempting to speculate that impaired Ca²⁺ signals contribute to the immune dysregulation observed in patients and mice lacking these proteins.

8. Conclusion

Inborn errors of CRAC channel function in patients with autosomal recessive mutations in the CRAC channel gene *ORAI1* and that of its activator *STIM1* are defined by a unique immunodeficiency syndrome. Its phenotype provides essential information about the physiological function of CRAC channels. In combination with studies in *Orai1*, *Stim1* and *Stim2* deficient mice, these patients have established an important role for ORAI1 and STIM1 in adaptive and innate immune responses to infection, in skeletal muscle function and ectodermally derived tissues such as teeth and sweat glands (**Figure 3**). CRAC channels have important functions in additional tissues and cell types, although no overt clinical pathologies have been observed in ORAI1 and STIM1 deficient patients yet that indicate an abnormal function of, for instance, pancreatic acinar, smooth muscle or endothelial cells⁴³. It is possible that CRAC channels play a somewhat redundant role in these cell types and that disease phenotypes only emerge under conditions when other, potentially compensating Ca²⁺ signaling pathways start to fail. Strong evidence in favor of a critical role for SOCE outside the immune system comes from mice with genetic deletion of *Orai1* and *Stim1* which are perinatally lethal^{63, 64, 75, 82, 86, 93}. Since deletion of both genes results in a similar phenotype, it is likely that the cause of death is related to impaired SOCE and not an exclusive, non-SOCE related function of ORAI1 or STIM1 proteins. The cause of death in these mice is unclear but occurs too early to be explained by immune dysregulation. Additional research will also have to address the physiological function of ORAI2 and ORAI3 *in vivo*, which is poorly defined. Important clues may come from the genetic deletion of these genes in mice or the discovery of patients with mutations in *ORAI2* and *ORAI3* genes. Both genes may have functions within the immune system that need to be explored. SOCE has been implicated in the function of many innate immune cells and it will be important to understand how impaired SOCE in these cells contributes to the immunodeficiency of ORAI1 and STIM1 deficient patients. Finally, the fact that the clinical phenotype in these patients is relatively limited to the immune system and studies in mice showing that SOCE is required for T-cell dependent autoimmune responses^{82, 85, 87} suggest that CRAC channels may be a good drug target for immune modulation in the context of inflammation. Given the almost ubiquitous expression of ORAI and STIM genes, the observation of CRAC currents in many cell types and the perinatal lethality of *Orai1* and *Stim1* deficient mice, more research is, however, required to understand the physiological role of SOCE *in vivo*.

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Figure 1. Store-operated Ca²⁺ entry (SOCE) through CRAC channels and its functions in immune cells

SOCE in T, B, NK and mast cells, neutrophils and macrophages is induced by engagement of immunoreceptors such as the TCR, BCR, Fc and Fc receptors and activating NK cell receptors. The activation of PLC 1 and PLC 2 results in the production of InsP₃ and the release of Ca²⁺ from ER Ca²⁺ stores via the opening of InsP₃ receptor channels. The resulting decrease of the [Ca²⁺]_{ER} is sensed by STIM1 and STIM2, which translocate to ER-plasma membrane junction where they bind to and activate ORAI1 CRAC channels. The resulting sustained Ca²⁺ influx activates Ca²⁺ regulated enzymes such as calcineurin and is required for the transcription of many genes (especially cytokine genes regulated by the Ca²⁺/calcineurin dependent transcription factor NFAT). SOCE is necessary for the degranulation and cytotoxic function of CD8⁺ T cells and NK cells, phagocytosis by macrophages, and production of reactive oxygen species in neutrophils. Abbreviations: CRAC, Ca²⁺ release activated Ca²⁺ channels; ER, endoplasmic reticulum; InsP₃, inositol-1,4,5-trisphosphate; NFAT, nuclear factor of activated T cells; PLC, phospholipase C; STIM, stromal interaction molecule.

Figure 2. Mutations in *ORAI1* and *STIM1* genes abolish SOCE and cause immunodeficiency
Autosomal recessive mutations in the human *ORAI1* and *STIM1* genes abolish CRAC channel function by interfering with ORAI1 or STIM1 mRNA expression (red), protein expression (orange) or their function (green). Protein domains in STIM1 are shown as shaded boxes (abbreviations: CC, coiled-coil domain; EFh, EF hand domain; SAM, sterile alpha motif; S/P, serine/proline rich). For details see text.

Figure 3. Synopsis of the clinical phenotype in CRAC channelopathy

Autosomal recessive mutations in *ORAI1* and *STIM1* are associated with a unique clinical phenotype that is characterized by (1) severe immunodeficiency (despite normal leukocyte numbers), (2) autoimmune cytopenias and hepatosplenomegaly / lymphadenopathy, (3) congenital, global muscular hypotonia and partial iris hypoplasia, (4) ectodermal dysplasia (anhidrosis and hypocalcified amelogenesis imperfecta, type III). Muscular hypotonia correlates histologically with a predominance of slow twitch (type I) and atrophy of fast twitch (type II) muscle fibers. Upper panel: H&E stain of a muscle biopsy from a patient with *ORAI1*-R91W mutation; lower panel: immunofluorescence using anti-*ORAI1* and anti-MHC fast antibodies¹⁰. Common infections and pathogens in *ORAI1* and *STIM1* deficient patients are listed on the left (for details see Table 1). For a list of abbreviations see Table 1.

Table 1

Mutations and clinical phenotype of ORAI1 and STIM1 deficient patients.

Gene	ORAI1		STIM1	
Gene defect	R91W	A88S/X25	A103E / L194P	E128R/X9
Chromosome	12q24	12q24	12q24	11q15
Inheritance	AR	AR	AR (compound het.)	AR
# Patients	2	2	2	1
ORAI1/STIM1 expression and Ca ²⁺ influx				
mRNA / Protein	Yes / Yes	No / No	Yes / No	No / No
SOCE / ICRAC	absent / absent	absent / absent	absent / not tested	absent / not tested
Cell types affected	T cells, B cells, fibroblasts	T cells, fibroblasts	T & B cells, granulocytes, platelets, fibroblasts	B cells
Immunological Manifestations				
Immunodeficiency & Infections	BCG infection, rota virus infection, Interstitial pneumonia, gastrointestinal sepsis (P1)	Recurrent pneumonia, otitis, oral and gastrointestinal candidiasis, chronic diarrhea, pyelonephritis,	Pneumonia, diarrhea since birth, CMV infection.	Bacterial infections: UTI, sepsis, pneumonia (<i>E.coli</i> , <i>S.pneumoniae</i>); Viral infections (CMV, VZV, EBV)
Lymphoc. counts	Normal	Normal	Normal	Normal
Lymphoc. subsets	Normal	Normal	Normal	Foxp3+ Treg
T cell activation	Proliferation Cytokine production	Proliferation	Proliferation	Proliferation
Serum Ig levels	Normal - (no specific Ab)	Normal - (no specific Ab)	Normal - (no specific Ab)	Normal (no specific Ab)
Autoimmunity & Lymphoproliferation	No	Neutropenia, Thrombocytopenia	No	AIHA, Thrombocytopenia Splenomegaly, Lymphadenopathy
Extrainmunological Manifestations				
Myopathy	Yes	Yes	Yes	No
Ectodermal Dysplasia	Anhydrosis Enamel dentition defect	No	Anhydrosis Enamel dentition defect	No
Other	No	Idiopathic encephalopathy Facial dysmorphism, posterior arch closing defect	No	No
Outcome	Death from sepsis 11m (P1); Survival after HSCT, now 18y (P2)	Death from pneumonia 5m (P3); Death from encephalopathy, seizures, fever 11m (P4)	Death 8m (P5); EBV-associated post-HSCT lymphoproliferative disease, survival after two HSCT, now 18y (P6)	Death from HSCT complications 9y (P7); Death from encephalitis 18m (P8); Survival after HSCT, now 9y (P9)
References	10, 12, 59, 61, 76, 79, 96, 98	10, 78	10, 77	84, 88

Abbreviations: Ab, antibody; AIHA, autoimmune hemolytic anemia; AR, autosomal recessive; BCG, Bacille Calmette-Guérin; CMV, Cytomegalo virus; ICRAC, Ca²⁺ release activated Ca²⁺ (CRAC) channel current; GI, gastrointestinal; HSCT, hematopoietic stem cell transplantation; Ig, immunoglobulin; m, months; NT, not tested; P, patient; SOCE, store-operated Ca²⁺ entry; UTI, urinary tract infections; y, years.

* only abnormally sliced mRNA present.