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ORAI1 and STIM1 deficiency in human and mice: roles of store-operated Ca^{2+} entry in the immune system and beyond

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Summary:

Store-operated Ca^{2+} entry (SOCE) is a mechanism used by many cells types including lymphocytes and other immune cells to increase intracellular Ca^{2+} concentrations to initiate signal transduction. Activation of immunoreceptors such as the T-cell receptor, B-cell receptor, or Fc receptors results in the release of Ca^{2+} ions from endoplasmic reticulum (ER) Ca^{2+} stores and subsequent activation of plasma membrane Ca^{2+} channels such as the well-characterized Ca^{2+} release-activated Ca^{2+} (CRAC) channel. Two genes have been identified that are essential for SOCE: ORAI1 as the pore-forming subunit of the CRAC channel in the plasma membrane and stromal interaction molecule-1 (STIM1) sensing the ER Ca^{2+} concentration and activating ORAI1-CRAC channels. Intense efforts in the past several years have focused on understanding the molecular mechanism of SOCE and the role it plays for cell functions *in vitro* and *in vivo*. A number of transgenic mouse models have been generated to investigate the role of ORAI1 and STIM1 in immunity. In addition, mutations in ORAI1 and STIM1 identified in immunodeficient patients provide valuable insight into the role of both genes and SOCE. This review focuses on the role of ORAI1 and STIM1 *in vivo*, discussing the phenotypes of ORAI1- and STIM1-deficient human patients and mice.

Keywords

ORAI1; STIM1; Ca^{2+}; T cells; B cells; mast cells

Introduction

Modulation of intracellular Ca^{2+} levels provides a signal transduction mechanism that is used by virtually all cells types – including lymphocytes – for the control of both short-term and long-term cellular functions. In the immune system, engagement of immunoreceptors such as the T-cell receptor (TCR), B-cell receptor (BCR), or Fc receptors on mast cells, dendritic cells, and macrophages results in a robust influx of Ca^{2+} from the extracellular space (1, 2). This influx in many instances is due to store-operated Ca^{2+} entry (SOCE) mediated by SOC channels of which the Ca^{2+} release-activated Ca^{2+} (CRAC) channel is the best characterized. While other pathways for Ca^{2+} influx may co-exist with SOCE in cells of the immune system, SOCE and the prototypical store-operated CRAC

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channel play a prominent role in Ca^{2+} signaling. In fact, the CRAC channel current I_{CRAC} was first described in T cells and mast cells (3, 4). SOCE, by definition, is activated by the depletion of endoplasmic reticulum (ER) Ca^{2+} stores that in immune cells generally occurs following antigen recognition, immunoreceptor tyrosine-based activation motif (ITAM) phosphorylation, phospholipase C (PLC) activation, and production of inositol-1,4,5-triphosphate (IP3). Binding of IP3 to IP3 receptors (IP3Rs) in the ER membrane induces Ca^{2+} efflux from the ER through IP3R resulting in (i) a transient increase in intracellular Ca^{2+} concentrations and (ii) activation of SOC channels in the plasma membrane. Major break-throughs in the understanding of SOCE have been made in the past few years with the discovery of stromal interaction molecule 1 (STIM1) and ORAI1 (or CRACM1) as essential molecular components of this pathway (5). Their identification has resulted in a flurry of research describing both the mechanisms underlying CRAC channel activation and the role of SOCE in many tissues including the immune system. This review focuses mainly on the role of SOCE for immune function by describing insights gained from studying human patients with mutations in STIM1 and ORAI1 and mice with targeted deletion of *Stim1, Stim2*, and *Orai1*.

Mechanisms and roles of Ca^{2+} signaling in the immune system

Ca^{2+} signals contribute to the function of many cell types in the immune system, including T and B cells, natural killer (NK) cells, mast cells, dendritic cells, and macrophages, where they control to diverse cell functions ranging from differentiation, proliferation, gene expression, cell motility, to secretion of vesicles containing cytokines, cytotoxic, or proinflammatory proteins (2, 6). In immune cells, Ca^{2+} signals result from engagement of antigen receptors at the cell surface and therefore directly determine the strength of the immune response to antigen. In addition, Ca^{2+} signals can be initiated through cell surface molecules and coreceptors such as CD19, CD20, and CD81 on B cells (7–9) and G-protein-coupled chemokine receptors on a variety of immune cells including dendritic cells (10). Details of how these receptors induce Ca^{2+} signals and which Ca^{2+} channels are activated is not well understood in many cases, although CRAC channel currents were observed in neutrophils in response to interleukin-8 (IL-8) treatment (11) and dendritic cells following passive store depletion (12). Other channels that may be involved in Ca^{2+} influx in immune cells include TRP (transient receptor potential) channels (13, 14), the adenosine triphosphate (ATP)-responsive P2Y and P2X purinoreceptors expressed on T cells and mast cells (15–18), and – although controversial – voltage-gated Ca^{2+} channels (19, 20). While TRP channels have not been shown to function as Ca^{2+} -selective cation channels in T cells, it is of note that TRPC1 could be co-immunoprecipitated together with STIM1 and ORAI1 in HEK293 and salivary gland cells (22) and that STIM1 was shown to gate TRPC1 and other TRPC channels (21). TRPM4, by contrast, functions as a calcium-activated non-selective cation channel that regulates Ca^{2+} influx in T cells and dendritic cells by effecting plasma membrane depolarization (23, 24). In a similar manner, potassium channels such as the voltage-sensitive Kv1.3 and the Ca^{2+} sensitive IKCa1 channel do not themselves conduct Ca^{2+} but are essential for Ca^{2+} entry by maintaining a negative membrane potential and providing the electrical driving force required for Ca^{2+} influx. As a consequence, inhibition of K^+ channel function suppresses Ca^{2+} entry and T-cell activation (25). The strength and

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duration of cytosolic Ca^{2+} signals is limited by re-uptake of Ca^{2+} into the ER or export into the extracellular space by Ca^{2+} pumps (26). B cells in addition use a variety of inhibitory receptors and signaling mechanisms to attenuate Ca^{2+} signaling through molecules such as SHP-1, CD22, Dok-3, Grb2, PD-1 and FcγRIIB in B cells (27–29).

Ca^{2+} signals are involved in a multitude of short- and long-term functions of immune cells (1, 2, 6). These include the regulation of T-cell motility during TCR-mediated antigen recognition on antigen-presenting cells (APCs) and formation of the immunological synapse (30–32), secretion of vesicles in mast cells and cytotoxic T cells (33), and phagocytosis in neutrophils (34) and macrophages (35–37). In mast cells, Ca^{2+} signals were shown to be involved in FcεRI-mediated degranulation and the release of histamine, leukotrienes, and prostaglandins (38–41). A similar process, the exocytosis of cytolytic granules by $CD8^{+}$ cytotoxic T cells, also depends on Ca^{2+} influx (33). When $CD8^{+}$ T cells recognize virus-infected or tumor cells, they form a synapse-like structure with their target cell that allows for the directional secretion of cytolytic granules containing perforin and granzyme proteases, resulting in the induction of apoptosis or necrosis in target cells (42). In the absence of Ca^{2+} influx, granule exocytosis and target cell apoptosis are impaired (33, 43).

Ca^{2+} signals are also involved in more long-term processes such as the regulation of cytokine and chemokine gene expression through Ca^{2+} -dependent transcription factors such as nuclear factor for activation of T cells (NFAT) (reviewed in 44–47) and certain cell fate decisions. The differentiation of naive $CD4^{+}$ T cells into T-helper 1 or 2 cells was shown to depend on the strength of signals mediated by the TCR including Ca^{2+} signals (48), and sustained increases in intracellular Ca^{2+} in the presence or absence of costimulatory signals are involved in the decision whether a T cell is activated or becomes unresponsive to future TCR stimulation (reviewed in 49–51). The role of Ca^{2+} signals, particularly SOCE, for T-cell development is discussed in more detail further below in the context of human patients and mice lacking SOCE (52). Taken together, Ca^{2+} influx is critically involved in many effector functions and cell-fate decisions controlling adaptive and innate immune responses. The absence of Ca^{2+} influx through SOCE results in immunodeficiency and autoimmunity, as discussed in detail further below.

Molecules mediating SOCE: the STIM and ORAI protein families

STIM1 and STIM2

STIM1 is a single-pass transmembrane protein, which is localized predominantly in the membrane of the ER where it functions as a sensor of ER Ca^{2+} concentrations and essential activator of ORAI1/CRAC channels (53, 54). The N-terminus of STIM1 contains a pair of low-affinity EF hand calcium-binding domains adjacent to a sterile α motif (SAM) protein–protein interaction domain; the longer C-terminus of STIM1 features two coiled-coil domains, serine/proline-rich, and lysine-rich domains (68, 69). Depletion of Ca^{2+} from the ER results in dissociation of Ca^{2+} from the N-terminal EF hand domains of STIM1, unfolding of the EF-SAM domain, and multimerization of STIM1, ultimately leading to the assembly of STIM1 in large clusters in the ER membrane, which are conventionally called puncta (55, 56). STIM1 puncta formation causes aggregation of ORAI1 in the plasma membrane and was shown to coincide with localized Ca^{2+} influx (56–58). Multimerization

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of STIM1 in the ER is sufficient to activate CRAC channels, elegantly demonstrated by substituting the ER-luminal N-terminus of STIM1 with artificial inducible protein–protein interaction domains (59). Expression of the cytoplasmic C-terminus of STIM1 as a soluble protein, however, is also able to constitutively activate CRAC channels in the absence of ER store depletion (60) and to induce assembly of ORAI1 into functional tetrameric complexes (61, 62).

Several laboratories including ours have now identified a minimal CRAC channel activation domain within the C terminus of STIM1 (63–66). This domain, when expressed by itself, is sufficient to colocalize with and bind to ORAI1 and activate the CRAC channel. We found that coiled-coil domain containing fragment b9 (CCb9) encompassing amino acids 339–446 is sufficient for binding to the C-terminus of ORAI1 and activation of CRAC channels in the absence of store depletion (66). Minimal activation domains identified by other groups are very similar in extent and comprise amino acids 342–448 [CRAC channel activation domain(CAD)](64), 344–442 [STIM1 Orai activating region (SOAR)](62), and 233–450/474 [Orai1-activating small fragment (OASF)] (67). Further truncation of the CCb9 fragment [and CAD (64)] by 10 amino acids at its C-terminal end resulted in a non-activating fragment (CCb10). Some minimal activation domains were shown to form tetramers and cluster ORAI1, but clustering itself does not seem to be sufficient for CRAC channel activation (63, 64). The CCb9 minimal activation domain is flanked at its C-terminal end by an approximately 31 amino acid peptide (445–475) that when applied directly to the cytoplasm inhibits CRAC channel activation (66). This finding suggests that the STIM1 minimal activation domain is masked in the context of full-length protein under resting conditions (i.e., replete Ca^{2+} stores) by an adjacent inhibitory peptide. This inhibition is presumably released when STIM1 undergoes conformational changes following depletion of Ca^{2+} stores (Fig. 1). Taken together, many new details regarding the structure and functional domains of STIM1 and their interactions with ORAI1 are emerging that elucidate the mechanisms involved in CRAC channel activation.

STIM2 is a closely related paralogue of STIM1 that shares its overall protein domain architecture including the EF hand Ca^{2+} binding, SAM, and coiled-coiled domains (68). STIM2, like STIM1, is located in the ER and was shown to heterodimerize with STIM1 (68). Early experiments on the function of STIM2 using RNA interference (RNAi)-mediated knock-down of STIM2 showed either no or only moderate effects on Ca^{2+} influx in HeLa or HEK293 cells in contrast to knock-down of STIM1 (53, 54). While one study overexpressing STIM2 suggested that it may have a potential inhibitory effect on STIM1-mediated store-operated Ca^{2+} influx (70), the majority of evidence now indicates that STIM2 is a positive regulator of SOCE and CRAC channel activation. STIM2 activates Ca^{2+} influx upon smaller decreases in ER Ca^{2+} concentrations than STIM1 and was suggested to regulate basal cytosolic Ca^{2+} concentrations (71). These data are consistent with a lower affinity of the EF hand Ca^{2+} -binding domain in STIM2 compared to that of STIM1. Analyzing T cells from mice lacking STIM2 expression, we observed that *Stim2*-deficient T cells have almost normal Ca^{2+} influx and CRAC channel function in the first few minutes following T-cell activation (72). They fail, however, to sustain increased intracellular Ca^{2+} concentrations for prolonged periods of time after store depletion compared with control cells. As a consequence, nuclear translocation of the Ca^{2+}-dependent transcription factor

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NFAT is rapidly reversed resulting in impaired NFAT-dependent cytokine gene expression in *Stim2*-deficient T cells despite normal expression of STIM1 (72). This delayed role of STIM2 in the regulation of store-operated Ca^{2+} influx relative to STIM1 is consistent with the much slower unfolding kinetics of the EF-SAM domain in STIM2 compared with STIM1 (74). These findings suggest that STIM1 and STIM2 respond with different thresholds and different activation kinetics to ER Ca^{2+} store depletion but also that both molecules synergize in their regulation of store-operated Ca^{2+} entry. This synergy is apparent in (i) the ability of STIM2 – when overexpressed – to restore SOCE in cells from human patients and mice lacking STIM1 (72, 75) and (ii) the more severe phenotype in mice with T-cell-specific deletion of both *Stim1* and *Stim2* compared with individual knockout mice (72).

ORAI1, ORAI2, and ORAI3

ORAI1 (or CRACM1) was identified as a regulator of Ca^{2+} signaling in RNAi screens and as the gene responsible for immunodeficiency in patients with a defect in CRAC channel function (76–78). The *ORAI1* gene on human chromosome 12q24 encodes an evolutionarily highly conserved tetraspanning plasma membrane protein that is structurally unrelated to other proteins or ion channels. Two paralogues ORAI2 (or CRACM2) and ORAI3 (or CRACM3) (79) are encoded by genes on human chromosomes 7q22 and 16p11, respectively. Strong functional and genetic evidence indicates that ORAI1 functions as the pore-forming subunit of the CRAC channel (80–82). Two negatively charged glutamate residues in the first and third transmembrane domain of ORAI1, E106, and E190, are thought to act as Ca^{2+} -binding sites in the ion channel pore. Mutations in either glutamate residue do not interfere with protein expression but abolish or significantly alter CRAC channel function (80–82). The native CRAC channel is characterized by its high Ca^{2+} selectivity and other distinctive properties including an inwardly rectifying current–voltage (I–V) relation and very low single-channel conductance (approximately 1 pS) (83). ORAI1-CRAC channels mutated at residues E106 or E190 cease to selectively conduct Ca^{2+} but become permeable to Na^{+} and Cs^{+}, presumably by interference of the mutations with Ca^{2+} binding in the selectivity filter of the CRAC channel pore. Recent studies indicate that functional CRAC channels likely consist of ORAI1 tetramers (61, 62, 84) reminiscent of voltage-gated Ca^{2+} channels. In this model, it is likely that each ORAI1 subunit contributes one or two glutamate residues for coordinated Ca^{2+} binding in the CRAC channel pore.

ORAI2 and ORAI3 share the predicted tetraspanning membrane topology with ORAI1 and show a high degree of sequence identity when compared with full-length ORAI1 (60.3% for ORAI1 and ORAI2, 63.2% for ORAI1 and ORAI3, and 66.4% for ORAI2 and ORAI3). Sequence homology is almost complete when comparing only the transmembrane domains (92.5%, 93.8%, and 93.8%, respectively). Both ORAI2 and ORAI3 form Ca^{2+} -permeable ion channels when overexpressed *in vitro* together with STIM1 (85, 86). The biophysical properties of currents recorded under these conditions are similar to native I_{CRAC} and those observed in cells co-expressing ORAI1 and STIM1. They differ, however, from ORAI1-mediated and native CRAC channel currents in a number of respects, for instance Ca^{2+} -dependent inactivation, monovalent permeation, and responsiveness to 2-APB (85, 86). These findings suggest that ORAI2 and ORAI3 may play a role in CRAC channel

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function and store-operated Ca^{2+} entry *in vivo*. No direct evidence for endogenous ORAI2 and ORAI3 function currently exists, however, and the tissues and cell types in which the two proteins may play a role remain to be determined.

STIM and ORAI expression in cells of the immune system

SOCE and CRAC channel activity have been documented in a variety of immune and non-immune cells (reviewed in 87). Outside the immune system, these cells include (but are not limited to) vascular endothelial (88–90) and smooth muscle cells (91, 92), pancreatic acinar cells (93), hepatocytes (94), and adrenal chromaffine cells (95). In the immune system, SOCE and I_{CRAC} have been observed in many different cell types of the lymphoid and myeloid lineage such as T cells (3), mast cells (4), B cells (96–98), dendritic cells (12), macrophages (99), NK cells (100), and neutrophils (11). It is likely that SOCE plays a role in other cell types in the immune system as well and contributes to proper innate and adaptive immune responses.

With the cloning of the STIM and ORAI gene families, expression data can be added to the picture to explain in which cells SOCE and CRAC channels are functional. mRNA for STIM1 and STIM2 is widely expressed in many tissues in both human and mouse including cells of the immune system (68, 69, 101) (Fig. 2). Human STIM1 and STIM2 are strongly expressed in lymphoid and myeloid cells such as T cells, B cells, NK cells, and monocytes (101, 102). In mice, STIM1 mRNA levels are the highest in mast cells, NK cells, and T cells, whereas STIM2 expression is fairly uniform and average in immune cells compared with other organs (101, 102) (Fig. 2). Using a transgenic expression system, Stiber *et al.* (103) detected STIM1–LacZ fusion protein in skeletal muscle, cerebellum, spleen, and thymus of mice heterozygous for the transgene. We observed strong STIM1 protein expression in naive and differentiated murine $CD4^+$ T cells (72), whereas STIM2 was undetectable in naive $CD4^+$ T cells and upregulated only upon differentiation into Th1 and Th2 cells *in vitro*. Expression levels of STIM2 were low, however, compared with those of STIM1 in both naive and differentiated $CD4^+$ T cells (72).

All three ORAI paralogues are expressed in a large variety of human and mouse tissues and cell types including lymphoid organs (101, 102, 104, Stefan Feske, unpublished data) (Fig. 2). Generally, the expression of human ORAI1 and ORAI3 mRNA is more ubiquitous than that of ORAI2, which in human is predominantly expressed in kidney, lung, and spleen (104). Murine ORAI1, ORAI2, and ORAI3 transcripts are found in most myeloid and lymphoid cells such as macrophages, dendritic cells, mast cells, T cells, and B cells (101, 102, 105) (Fig. 2). Particularly, high levels of ORAI1 mRNA were observed in mouse granulocytes, whereas ORAI2 and ORAI3 expression was strongest in lipopolysaccharide (LPS)-stimulated macrophages and mast cells, respectively (101) (Fig. 2). Interestingly, ORAI2 expression is high in naive $CD4^+$ T cells (38, 106) and is downregulated upon differentiation into Th1 or Th2 cells (106). This led to the suggestion that ORAI2, not ORAI1, is responsible for SOCE in naive T cells (38).

Expression data on ORAI proteins is very limited due to the current lack of reliable commercial antibodies against ORAI1, ORAI2, and ORAI3. Using a custom-made ORAI1-

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antibody, we evaluated the distribution of ORAI1 protein expression in human and mouse tissues including primary and secondary lymphoid organs (Stefan Feske, unpublished data). In human lymphoid organs, ORAI1 was detected in a subset of cells in the thymus, spleen, and tonsils by immunohistochemistry. The highest expression was seen in cells of the periarterial lymphoid sheath (PALS) of the spleen and the paracortical zone in tonsils, consistent with ORAI1 expression in T cells. In mice, ORAI1 protein was detected fairly uniformly in all lymphocyte populations tested including $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, and NKT cells isolated from spleen and lymph nodes (Fig. 3). No significant differences in expression were detected between T-cell subsets including effector T cells, regulatory T cells (defined as $CD4^+CD25^+GITR^+$), naive and memory $CD4^+$ and $CD8^+$ T cells, or between resting ($CD69^-$) and stimulated ($CD69^+$) T cells (Fig. 3). ORAI1 protein was equally expressed at all stages of T-cell development in the thymus (Fig. 3). Similar protein expression data for ORAI2 and ORAI3 are not available. Taken together, both STIM1 and ORAI1 are expressed almost ubiquitously in human and mouse tissues and many if not all cells of the immune system.

CRAC-channelopathies due to mutations in ORAI1 and STIM1 in human patients

Mendelian diseases caused by mutations in single genes have provided important insights into the function of many genes based on the pathophysiology associated with absent, nonfunctional, or dominant negative mutant gene products. Early evidence for the function and crucial role of SOCE and CRAC channels *in vivo* came from the analysis of patients with rare but very instructive inherited immunodeficiency diseases (78, 107–109). Patients lacking store-operated Ca^{2+} influx and CRAC channel function show a severe defect in T-cell activation and suffer from immunodeficiency characterized by life-threatening viral, bacterial, and fungal infections. We have identified mutations in ORAI1 that interfere with CRAC channel function and STIM1 that generate a null phenotype by abolishing protein expression (75, 78). The clinical phenotypes of ORAI1 and STIM1 deficiency overlap to a large degree and are characterized by immunological and non-immunological symptoms, suggesting that the clinical syndrome associated with CRAC-channelopathy is pathway and not gene specific.

ORAI1 deficiency

Mutations and molecular characterization

ORAI1 was identified as the gene encoding the CRAC channel through the combination of linkage mapping in immunodeficient patients lacking SOCE and CRAC channel function (78, 110) and a genome-wide functional RNAi screen in *Drosophila melanogaster* S2 cells for genes regulating NFAT nuclear translocation and Ca^{2+} influx (76–78). Sequencing of the *ORAI1* gene revealed that immunodeficient patients are homozygous for a missense mutation in exon 1 of *ORAI1* that results in substitution of an arginine at position 91 of the protein sequence with tryptophane (R91W) (78) (Fig. 4). The single amino acid substitution abolishes CRAC channel function, SOCE, and T-cell activation but does not interfere with ORAI1 expression or its localization in the plasma membrane. R91 is located

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at the beginning of the first transmembrane domain of ORAI1, i.e., at the interface of cytoplasm and plasma membrane (Fig. 4). Substitution of R91 with other amino acids had variable effects on CRAC channel function with hydrophobic residues exerting the strongest inhibitory effect (111). The R91W mutation does not seem to interfere with interactions between ORAI1 and STIM1 (112, 113) and does not have a strong dominant negative effect on channel function. T cells isolated from heterozygous carriers of the R91W mutation showed partially impaired Ca^{2+} influx ranging from approximately 30–80% of that observed in wildtype control cells depending on the extracellular Ca^{2+} concentration (78). By contrast, expression of a single ORAI1-R91W mutant subunit together with three wildtype ORAI1 molecules in the context of a concatenated ORAI1 tetramer almost completely abolished CRAC channel function (114). Such a negative interfering effect of the ORAI1-R91W mutation would be significant because ORAI1 was shown to form functional homotetramers (61, 62, 84) and may form heteromultimers with ORAI2 and ORAI3 based on co-immunoprecipitation experiments (104). Whether such heteromultimers between ORAI1, ORAI2, and ORAI3 are formed *in vivo*, however, and whether the R91W mutation interferes with their function is unclear.

Clinical phenotype of ORAI1 deficiency

Lack of functional ORAI1 in human patients with ORAI1-R91W mutation is dominated clinically by immunodeficiency with severe infections early in life and, in addition, congenital myopathy and ectodermal dysplasia. Immunodeficiency is characterized by recurrent severe infections including rotavirus enteritis, BCGitis, pneumonia, meningitis, and gastrointestinal sepsis (107, 115, 116) (Table 1). Antibiotics and intravenous immunoglobulin (IVIg) only inefficiently controlled infections necessitating hematopoietic stem cell transplantation (HSCT). One patient survived after HSCT, while his older brother died before an appropriate donor could be found. Clinical symptoms of immunodeficiency in patients with mutations in ORAI1 are very similar to those observed in patients with severe combined immunodeficiency (SCID), although in contrast to the majority of SCID patients, absolute lymphocyte counts and numbers of $CD4^+$ and $CD8^+$ T cells and B cells were normal in ORAI1-deficient patients (Table 1). These findings suggest that lymphocyte development, positive selection of T cells in the thymus, and peripheral lymphocyte maturation are independent of ORAI1 or potentially SOCE. By contrast, activation of peripheral T cells is severely compromised, apparent in reduced or absent skin delayed-type hypersensitivity reactions *in vivo* and impaired proliferative responses to TCR-dependent and independent stimuli *in vitro* (107–109) (Table 1). While immunoglobulin levels were normal, ORAI1-deficient patients failed to mount antigen-specific antibody responses in response to vaccination and infections. Taken together, the immunodeficiency in ORAI1-deficient patients, despite its severity and life-threatening nature, is caused by the impaired function of T cells (and B cells) but not impaired lymphocyte development.

In addition to the defect in immune function, ORAI1 deficiency is characterized by congenital myopathy and ectodermal dysplasia. The myopathy manifests clinically very early in life as global muscular hypotonia with decreased head control, delayed ambulation, reduced muscle strength and endurance (77, Stefan Feske, unpublished data). Chronic pulmonary disease is likely to result from impaired respiratory muscle function. The

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ectodermal dysplasia in ORAI1-deficient patients is characterized by impaired sweat production and a defect in dental enamel formation. Sweat provocation tests in the patient homozygous for the ORAI1-R91W mutation were abnormal, and the reduced sweat production results in dry skin and heat intolerance with recurrent fever, especially in the summer. The severely dysplastic dental enamel matrix is hypocalcified, resulting in painful exposure of the underlying yellow dentin. The phenotype is consistent with the diagnosis of ectodermal dysplasia with anhydrosis (EDA), although other symptoms of EDA such as sparse scalp hair or eyebrows were not present. The clinical phenotype of EDA suggests that SOCE mediated by ORAI1 is involved in Ca^{2+} transport in eccrine sweat glands and that SOCE is required for calcification of the dental enamel matrix.

Defects in SOCE and CRAC channel function were reported in additional patients from two unrelated families suffering from congenital immunodeficiency (108, 109). Patients from both families suffered from severe T-cell immunodeficiency with recurrent viral, fungal, and bacterial infections similar to the individuals with ORAI1-R91W mutation. SOCE was severely impaired in lymphocytes of all patients; in addition, I_{CRAC} was undetectable in one of the patients. The nature of the gene defect in these patients remained unknown, because ORAI1 and STIM1 had not been identified as mediators of SOCE at the time (108, 109).

STIM1 deficiency

Mutations and molecular characterization

Lack of STIM1 resulting from RNAi *in vitro* or genetic ablation of *Stim1* expression in mice *in vivo* results in a severe defect in CRAC channel activation and store-operated Ca^{2+} entry (53, 54, 72). We recently identified a homozygous nonsense mutation in *STIM1* as the cause for immunodeficiency in three patients from one family (75). Fibroblasts from one of the patients available for analysis showed a pronounced defect in SOCE in response to thapsigargin, an inhibitor of the sarco⁄endoplasmic reticulum Ca^{2+} ATPase (SERCA), which induces passive depletion of intracellular Ca^{2+} stores. No mutations in *ORAI1*, *ORAI2*, and *ORAI3* were observed in the index patient or her younger brother (no DNA of the third patient was available for analysis). Instead, both patients were homozygous for an insertion of an adenine in exon 3 of *STIM1* resulting in a frameshift and premature termination at position 136 of STIM1 protein (E136X, or E128RfsX9 as the mutation leads to a frame shift beginning at codon 128 and a STOP codon nine amino acids later). We failed to detect either full-length STIM1 or the predicted N-terminal STIM1 fragment in the patients cells. Strongly reduced STIM1 mRNA transcript levels indicated that the nonsense mutation likely results in nonsense-mediated mRNA decay and a STIM1 null-phenotype. SOCE was rescued by reintroducing STIM1 into the patients' cells, whereas expression of ORAI proteins had no effect. Ectopic expression of STIM2 was able to partially rescue SOCE, suggesting that STIM1 and STIM2 have overlapping functions; nevertheless, endogenous expression levels of STIM2 do not seem to be sufficient to compensate for the lack of STIM1 in the patients' cells.

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Clinical phenotype of STIM1 deficiency

Lack of STIM1 expression is characterized clinically by immunodeficiency, congenital myopathy, and ectodermal dysplasia reminiscent of the phenotype observed in ORAI1-deficient patients, and, in addition, autoimmune disease (75)(Table 1). The immunodeficiency in STIM1-deficient patients is marked by recurrent bacterial and viral infections. The index patient suffered from urinary tract infections, otitis media, pneumonia and multiple episodes of sepsis, caused by a spectrum of pathogens such as *Streptococcus pneumoniae*, *Escherichia coli*, cytomegalovirus, and varizella zoster virus. Her younger sister had died from encephalitis and enteroviral infection at the age of 18 months, and a younger brother suffered from sepsis during the first 2 months of his life before receiving HSCT. Total lymphocyte counts and numbers of T cells, B cells, and NK cells were normal in the patients' blood as was the TCR repertoire in T cells of the index patient. T cells of two of the patients, however, showed a severe defect in proliferation *in vitro*. Serum Ig levels for all subtypes were mostly normal in two of the patients, but strongly reduced IgG levels were found in the third patient due to nephrotic syndrome. Taken together, STIM1-deficient patients – like those lacking functional ORAI1 – do not show a gross defect in lymphocyte development but are severely compromised in T-cell activation.

Immunodeficiency in all three STIM1-deficient patients was complicated by the presence of lymphoproliferative and autoimmune disease (75). Two of the patients showed lymphadenopathy and hepatosplenomegaly despite normal Fas-induced apoptosis measured in T cells of one patient. All three patients presented with thrombocytopenia and two suffered from Coombs-positive autoimmune hemolytic anemia (AIHA). The thrombocytopenia, like the anemia, is due to autoimmunity and not a defect in platelet development, because platelets were coated with autoantibodies against platelet glycoprotein Ib/IX and platelet numbers recovered following glucocorticoid therapy but not in response to platelet transfusions. A likely cause for the autoimmunity in the STIM1-deficient patients is the reduced number of $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells (Tregs) found in the peripheral blood of the index patient (blood samples from the other patients were not available for analysis). This situation is reminiscent of mice with T-cell-specific deletion of both *Stim1* and *Stim2*, which show greatly reduced numbers and impaired function of Treg cells (72). The mice display many signs of an autoimmune lymphoproliferative phenotype such as splenomegaly, lymphadenopathy, leukocytic organ infiltration, dermatitis, and blepharitis. In the absence of *STIM1* expression in humans and that of *Stim1* and *Stim2* in mice, SOCE is abolished and with it the activation of the Ca^{2+} -regulated transcription factor NFAT (72). Binding sites for NFAT exist in the promoter of the Treg-lineage specific transcription factor Foxp3 (73), and NFAT was shown to form a complex with Foxp3 at a composite DNA-binding site in the IL-2 promoter (117). In the absence of SOCE and NFAT activation, Foxp3 expression is presumably reduced, and NFAT:Foxp3 complexes are not formed providing a potential explanation for the reduced numbers of $Foxp3^{+}$ Treg cells in *Stim1/Stim2*-deficient mice and STIM1-deficient patients.

Despite reduced numbers of Tregs, STIM1-deficient patients did not present clinical features observed in X-linked immune dysregulation, polyendocrinopathy, enteropathy (IPEX) syndrome (118). The IPEX syndrome constitutes an immune disorder due to

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Foxp3 deficiency and is characterized by severe enteropathy and autoimmune diabetes. The absence of more severe IPEX-like symptoms in STIM1-deficient patients may be explained by the impaired antigen-specific activation of effector T cells in the absence of STIM1 and SOCE. While the reduced numbers of Treg cells are likely to be the main cause of the autoimmune phenotype in the STIM1-deficient patients, we cannot exclude that in addition lack of SOCE in developing T cells results in abnormal checkpoint control of self-reactive T cells during negative selection in the thymus. The severe defect in T-cell activation and immunodeficiency observed in STIM1-deficient patients is very similar to that seen in ORAI1-deficient patients with the notable exception of autoimmunity and reduced numbers of Treg cells.

In addition to immunodeficiency and autoimmunity, STIM1-deficient patients suffer from ectodermal dysplasia and congenital myopathy reminiscent of patients lacking functional ORAI1. Ectodermal dysplasia is characterized by a severe defect in dental enamel formation. Myopathy presented as non-progressive global muscular hypotonia and partial iris hypoplasia. A muscle biopsy and electromyography did not show abnormalities indicative of common neuropathies or myopathies. The myopathy observed in the STIM1-deficient patients correlates well with that observed in ORAI1-deficient patients, and the defect in skeletal muscle development and function found in *Stim1*-deficient mice (103). The myopathy is consistent with the function of ORAI1 and STIM1 in store-operated Ca^{2+} influx in skeletal muscle and the demonstration that STIM1 is required for myoblast differentiation (103, 119, 120).

Comparison of ORAI1 and STIM1 deficiency

The clinical phenotypes of ORAI1- and STIM1-deficient patients indicate that ORAI1 and STIM1 play important roles in CRAC channel function and SOCE during T-cell activation, skeletal muscle development and/or function and ectodermal derived tissues such as teeth and sweat glands (Table 2). The clinical phenotypes associated with defects in both genes overlap to a large degree, suggesting that they are not gene but pathway specific, i.e. that they result from the absence of SOCE and CRAC channel function. The severity of immunodeficiency and the spectrum of pathogens causing infections are similar in patients lacking functional ORAI1 and STIM1. Importantly, the immunodeficiency in both cases is characterized by a severe defect in T-cell activation but not a gross defect in lymphocyte development. T cells failed to proliferate *in vitro* and showed impaired skin delayed type hypersensitivity reactions *in vivo*. By contrast, total lymphocyte counts and numbers of T, B, and NK cells were normal in ORAI1- and STIM1-deficient patients, suggesting that SOCE mediated by neither gene is required for T-cell development. Although consistent with normal lymphocyte development in *Stim1*- and *Orai1*-deficient mice, this finding is surprising, because Ca^{2+} signals following TCR engagement are considered necessary for thymic T-cell development and selection. Potential explanations are discussed in more detail further below.

SOCE seems required however for the development of $CD4^{+}Foxp3^{+}$ Tregs, as indicated by the reduced numbers of Tregs in the peripheral blood of one STIM1-deficient patient and mice with T-cell-specific deletion of *Stim1* and *Stim2* resulting in lymphoproliferative

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disease and autoimmunity. Autoimmunity has not been observed in ORAI1-deficient patients and mice, and Treg numbers were normal in *Orai1*$^{-/-}$ mice (38, 78, 106); blood samples of ORAI1-deficient patients pre-HSCT were not available for analysis of Treg cells. A possible explanation for the differential effect of ORAI1 and STIM1 deficiency on Treg development and autoimmunity may lie in the distinct residual levels of SOCE in immature T cells. In mice, lack of both STIM1 and STIM2 completely abolished SOCE in naive T cells whereas residual Ca^{2+} influx was observed in naive T cells from Orai1$^{-/-}$ mice (106). It can be speculated that small amounts of SOCE are sufficient for Treg cell development.

Comparison of CRAC channelopathy with similar immunodeficiency syndromes

ORAI1 and STIM1-deficient patients suffer from very similar non-immunological symptoms including global muscular hypotonia and ectodermal dysplasia. The combination of immunodeficiency and ectodermal dysplasia was also observed in patients with hypomorphic mutations in NFκB essential modulator (NEMO) and a hypermorphic mutation in IκBα, in both cases impairing activation of the transcription factor NF-κB (121–123). These patients suffer from severe bacterial infections early in life, despite normal numbers and subset distribution of T, B, and NK cells. One patient with mutation of Ser32 in IκBα that prevents phosphorylation and degradation of IκBα lacked γ/δ T cells and memory α/β T cells. T-cell responses following TCR stimulation were significantly impaired. Similar to ORAI1- and STIM1-deficient patients, NEMO and IκBα mutant patients also suffered from EDA, which in their case is characterized by hypodontia and conical teeth. A dental enamel calcification defect and the myopathy present in SOCE-deficient patients were not observed, suggesting that the pathophysiology of disease in both groups of patients is different (123, 124).

Role of STIM1 and ORAI1 outside the immune system

SOCE and CRAC channel function have been reported in a variety of cell types outside the immune system years before the cloning of STIM and ORAI genes (reviewed in 87). Since then, SOCE was shown to require functional STIM1 and ORAI1 in a number of different cell types and tissues such as platelets, skeletal muscle, vascular endothelium, and smooth muscle (103, 125–128). An important role of both proteins outside the immune system is emphasized by the non-immunological symptoms observed in SOCE-deficient patients (75, 78, Stefan Feske, unpublished data) and mice (38, 39, 72, 105, 125, 127, 129). The disease spectrum in both ORAI1- and STIM1-deficient patients is limited to immunodeficiency, congenital myopathy, ectodermal dysplasia, and, in the case of STIM1 deficiency, autoimmunity (75, 78, Stefan Feske, unpublished data) (Tables 2 and 3). This does not, however, exclude a role for ORAI1, STIM1, and SOCE in other cell types or organs but rather points to a non-redundant role of these genes in the affected tissues. Potential reasons for the lack of more extensive disease in the patients include that (i) ORAI1 and STIM1 have partially redundant roles in SOCE and can be functionally replaced by ORAI2, ORAI3, and STIM2 or that (ii) SOCE co-exists with non-store operated Ca^{2+}influx in many other tissues compensating for the lack of functional ORAI1 and

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STIM1. As discussed earlier, *in vitro* studies showed that coexpression of ORAI2 and ORAI3 together with STIM1 results in large Ca^{2+} currents (85, 86), suggesting that both genes can form functional calcium channels. While *in vivo* evidence for a physiological role of endogenous ORAI2 and ORAI3 is currently missing, it is likely that both genes may play a role in SOCE in some cell types inside or outside the immune system.

In skeletal muscle, SOCE was described and both STIM1 and ORAI1 are expressed at high levels (38, 68, 106, 130). In addition, STIM1 and ORAI1 mediate SOCE in primary cultures of murine skeletal myotubes (103, 119) and are required for differentiation of human myoblasts from isolated satellite cells, the stem cells of adult skeletal muscle (120). Myotubes from *Stim1*-deficient mice have a contractile defect that is associated with rapid fatigue following repeated stimulation. Importantly, the majority of mice lacking functional STIM1 expression die early postnatally; surviving mice are runted and show a myopathy that was suggested to cause or contribute to the mortality in these mice (103). The myopathy observed in *Stim1*-deficient animals is consistent with the congenital myopathy in human patients lacking functional ORAI1 or STIM1(75, 78), and together these observations strongly suggest that SOCE mediated by STIM1 and ORAI1 plays a somewhat unexpected but important role in skeletal muscle function and development.

SOCE is required for the development and/or function of ectodermal derived tissues such as sweat glands and teeth given the EDA phenotype in patients with mutations in STIM1 and ORAI1 (75, 78, Stefan Feske, unpublished data). EDA is characterized mainly by hypocalcification of the dental enamel matrix and a defect in sweat production. Dental enamel is the most highly calcified tissue in the human body, but the mechanisms used by ameloblasts, enamel epithelial cells, to transport Ca^{2+} to the enamel matrix are poorly understood. Several ameloblast Ca^{2+} transport mechanisms have been proposed, including paracellular and transcellular routes; the latter may occur via the cytoplasm of amenoblasts or via the ER facilitated by SOCE (131). Direct experimental evidence for the ER route is missing, but it seems plausible given the defect in enamel calcification in STIM1- and ORAI1-deficient patients. The secretory activity of eccrine sweat glands is known to depend on Ca^{2+} influx (132, 133). SOCE has been reported in a variety of epithelia including equine epithelial sweat gland cells where it occurs at the basolateral membrane and is required for anion secretion (134). Given the expression of ORAI1 in human eccrine sweat glands and the defect in sweat production in ORAI1-deficient patients (78, Stefan Feske, unpublished data), SOCE seems to be required for sweat gland function. Roles for SOCE in other tissues and cell types mediated by STIM and ORAI proteins undoubtedly exist but cannot be discussed in this review.

Mouse models of ORAI1 and STIM1 function

Mice lacking *Orai1, Stim1*, and *Stim2* expression have been generated by a number of laboratories, and their phenotypes will be discussed here with an emphasis on the similarities and differences to human patients (Table 3). In contrast to ORAI1- and STIM1-deficient patients (75, 78), mice lacking expression of functional *Orai1* and *Stim1* genes die early postnatally with certain variations due to the method and genetic background used for gene-targeting. The precise cause of death is unclear, but morphological abnormalities

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in skeletal muscle of *Stim1*-deficient (103) and *Orai1* knock in mice (Stefan Feske, unpublished data) as well as functional defects in myoblasts of $Stim1^{-/-}$ mice (102) are likely to cause or contribute to their severely runted phenotype (38, 103, 106, 126, 127, Stefan Feske, unpublished data). The skeletal muscle defect in mice matches the congenital myopathy observed in human patients lacking functional ORAI1 and STIM1 (75, 78). In contrast to mice, the myopathy in humans is not life-threatening, and survival of ORAI1- and STIM1-deficient patients is limited by immunodeficiency and infections.

Role of ORAI1 for SOCE and immune function in mice

Lack of *Orai1* expression in mice strongly reduces the amplitude of CRAC currents in mast cells (38) and T cells (106), with residual currents observed in both cells types. As a consequence, SOCE is impaired in mast cells (38), B cells, and T cells (106) of $Orai1^{-/-}$ mice, although normal SOCE was observed in $CD4^+$ T cells in one study (38). A likely explanation for this discrepancy is that the SOCE defect was more pronounced in $CD4^+$ and $CD8^+$ T cells differentiated *in vitro* into Th1, Th2 cells, and cytotoxic T cells, respectively, compared with freshly isolated naive $CD4^+$ and $CD8^+$ T cells (38, 106, Stefan Feske, unpublished data). These findings suggest that regulation of SOCE changes during T-cell differentiation. Indeed, *Orai2* mRNA expression was observed in naive T cells from wildtype mice (38, 106) but decreased substantially upon differentiation into Th1 and Th2 cells (106). In naive mouse T cells, ORAI2 may therefore contribute to SOCE, whereas ORAI1 is the predominant ORAI family member in differentiated T cells (106). In human T cells, by contrast, ORAI1 is required for CRAC channel function given the complete absence of I_{CRAC} and SOCE in T cells from immunodeficient patients lacking functional ORAI1 (78). B cells from *Orai1*-deficient mice showed severely impaired SOCE (106, Stefan Feske, unpublished data). The defect was however more pronounced in response to passive store depletion with thapsigargin compared with BCR stimulation by anti-IgM crosslinking (106). This finding is consistent with the recently described ORAI1- and ORAI2-independent Ca^{2+} influx in response to BCR stimulation and suggests the presence of a non-store operated Ca^{2+} influx pathway in B cells (135).

In the absence of *Orai1* gene expression in mice, the function of mast cells, T cells, and B cells is compromised. Mast cell degranulation and cytokine secretion *in vitro* and passive cutaneous anaphylaxis *in vivo* are impaired in $Orai1^{-/-}$ mice (38) (Table 3). Furthermore, B cells proliferated poorly in response to BCR but not LPS stimulation in the absence of ORAI1, suggesting that Ca^{2+} signals are essential for antigen-dependent expansion of mouse B cells (106). Variable effects on T-cell function were observed in *Orai1*-deficient mice. Cytokine expression in $Orai1^{-/-}$ mice was substantially reduced for IL-2, IFN-γ, IL-4, and IL-10 in Th1 and Th2 cells, respectively (106) (Table 3). This finding is consistent with the greatly diminished cytokine gene expression found in T cells from human ORAI1-deficient patients (116). By contrast, no defect in T-cell proliferation or IL-2 and IFN-γ production was seen in another study (38). The cause of this discrepancy is not immediately clear but may have to do with the different methods used for gene targeting, genetic backgrounds of mice, or, most likely, the differentiation stage of T cells at the time of analysis.

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Remarkably, the development of lymphoid and myeloid cells such as T, B, and mast cells in the thymus and bone marrow appears to be unperturbed in the absence of ORAI1 in mice. T-cell development in the thymus and B-cell development in the bone marrow and spleen appeared normal in *Orai1*$^{-/-}$ mice (106). These findings are consistent with normal lymphocyte numbers and subsets observed in human ORAI1-deficient patients (38, 107) (Tables 1 and 3) as well as *Stim1*- and *Stim2*- deficient mice (72), suggesting that SOCE may be dispensable for lymphocyte development.

Role of STIM1 and STIM2 for SOCE and immune function in mice

Stim1-deficient mice, like those lacking functional *Orai1* expression, die early postnatally, and most studies have therefore been conducted using fetal liver chimeras (126, 136) or conditional T-cell-specific deletion of Stim1 and Stim2 using the Cre-loxP system (72). Lack of *Stim1* expression abolishes CRAC channel function and SOCE in mast cells, macrophages, and CD4$^+$ and CD8$^+$ T cells, both naive and *in vitro* differentiated (35, 39, 72). By contrast, deletion of *Stim2* in T cells had only a very moderate effect on SOCE and CRAC channel function, when tested in the first 5–10 min following T-cell stimulation but resulted in a marked defect in sustained Ca^{2+} influx and Ca^{2+} -dependent translocation of the transcription factor NFAT >10 min poststimulation, as discussed earlier (72). While STIM1 appears to play a greater role for CRAC channel activation than STIM2 in the immediate response to cell stimulation, STIM1 alone is not sufficient to maintain Ca^{2+} levels in *Stim2*-deficient T cells (72). A potential explanation is that STIM2 activation - compared to that of STIM1 - is (i) delayed due to the slower unfolding kinetics of its EFh-SAM domain and subsequent oligomerization (74) but (ii) maintained longer when calcium stores are refilling, thus contributing to sustained SOCE.

STIM1 is required for activation of mast cells, T cells, and macrophages (35, 39, 72), and presumably other immune cells as well. Mast cells lacking STIM1 were impaired in FscεRI-induced degranulation and cytokine production *in vitro* and showed an impaired passive cutaneous anaphylactic reaction *in vivo* (39), similar to observations made in *Orai1*-deficient mice (38). *Stim1*-deficient T cells also showed greatly reduced production of cytokines such as IL-2, IFN-γ, and IL-4, a defect that was also observed, although in milder form, in *Stim2*-deficient T cells, suggesting that both proteins are required for maintaining SOCE and inducing T-cell activation (72). It is likely that SOCE mediated by STIM1 is important for T-cell functions *in vivo* such as alloreactivity, control of viral infections, and anti-tumor responses by CD4$^+$ and CD8$^+$ T cells, although a recent study showed that T-cell-dependent antibody responses and graft-versus-host disease were comparable in *Stim1*-deficient and wildtype mice (136).

The role of Ca^{2+} influx for macrophage function has been controversial (36, 37, 137), but STIM1 and SOCE may play a role in FcRγ receptor-mediated activation of macrophages and phagocytosis *in vivo* (35). Murine macrophages lacking *Stim1* expression had severely compromised FcRγII/III-mediated Ca^{2+} influx (35) and were impaired in a number of *in vivo* models of autoantibody and immune complex induced macrophage function. Interestingly, when *Stim1*-deficient mice were injected with autoantibodies against platelets or red blood cells, they were protected from thrombocytopenia and anemia due to

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erythrophagocytosis by Kupffer cells in the liver. By contrast, human patients lacking STIM1 expression presented with the very diseases that *Stim1*-deficient mice were protected from, i.e., AIHA and thrombocytopenia (75). Apparently, lack of STIM1 and SOCE did not protect the patients' red blood cells and platelets from macrophage-mediated phagocytosis, pointing to a potential difference in the role of STIM1 for macrophage function in human and mouse.

While T-cell activation is profoundly impaired in the absence of STIM1 and ORAI1, T-cell development is unperturbed as numbers of T and B cells in the peripheral blood of ORAI1- and STIM1-deficient patients (75, 107–109), and T and B-cell development in the thymus and bone marrow of *Stim1*-, *Stim1/Stim2*-, and *Orai1*-deficient mice were normal (38, 72, 106, 136). This finding is unexpected, because TCR-induced Ca^{2+} signals have been considered necessary for proper T-cell development (138, 139). Mice with defective Ca^{2+} signaling such as *Slp76*, *Lat*, and *Itk* mutant mice (140–143) have blocks at various stages of T-cell development. Furthermore, positive selection of T cells in the thymus was impaired in mice lacking the regulatory B1 subunit of the Ca^{2+}-dependent phosphatase calcineurin (144) or the transcription factor NFAT4 (145, 146, reviewed in 45). Positive selection was also shown to be associated with Ca^{2+} oscillations in thymocytes (30). Most of this evidence is indirect, but collectively it suggested that SOCE as the main source of Ca^{2+} signaling in T cells is required for T-cell development.

Using *Stim1*-deficient mice (*Stim1*$^{fl/fl}$ *CMV-Cre*, crossed to ICR outbred mice to ameliorate early postnatal lethality), we found normal T-cell development in the thymus despite undetectable Ca^{2+} influx induced by TCR crosslinking or thapsigargin treatment in double negative, double positive, and single positive thymocytes (Masatsugu Oh-hora, Anjana Rao, Stefan Feske, unpublished data). Similar observations were made in other strains of Stim1$^{-/-}$ mice (38, 136) and in Orai1$^{-/-}$ mice (106). These findings are not easily reconciled with the idea that TCR-induced SOCE is required for T-cell development, as patients and mice lacking STIM1 and ORAI1 provide the most direct model systems to test this hypothesis. It can be speculated that ORAI1 is not required for store-operated Ca^{2+} influx in immature T cells, because ORAI2 and ORAI3 may mediate SOCE in those cells. However, T-cell development is also unperturbed in the absence of both STIM1 and STIM2 in double deficient mice (*Stim1*$^{fl/fl}$, *Stim2*$^{fl/fl}$ *CD4-Cre*) (72). If the widely accepted model that STIM1 and ORAI1 constitute the elementary unit of SOCE (5) is true, then SOCE is not required for T-cell development. In immature T cells, repeated release of Ca^{2+} from ER Ca^{2+} stores or influx through non-store operated channels such as TRP channels or P2 receptors may provide the Ca^{2+} signal necessary for T-cell development. Further studies are necessary to investigate the nature of Ca^{2+} signals in T-cell development.

In contrast to normal development of T and B cells, we observed a substantial defect in the development of $CD4^+$ $Foxp3^+$ regulatory T cells in human patients lacking STIM1 expression and mice with T-cell-specific deletion of both *Stim1* and *Stim2* (*Stim1*$^{fl/fl}$/*Stim2*$^{fl/fl}$ *CD4-Cre* mice) (72, 75). Treg numbers in the thymus, spleen, and lymph nodes of *Stim1/Stim2*-deficient mice were approximately 10% of those found in wildtype littermates. Reduced Treg cell numbers are due to an intrinsic defect in regulatory T-cell development as *Stim1/Stim2*-deficient Treg cells also failed to develop

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in the presence of wildtype Treg cells in mixed bone marrow chimeras (72). As discussed further above, a potential explanation for impaired development of Treg cells is the impaired Ca^{2+}-and NFAT-dependent induction of Foxp3 expression and the impaired formation of a NFAT:Foxp3 DNA-binding complex (73, 117). *Stim1*-deficient mice, in contrast to human *STIM1*-deficient patients, have normal Treg numbers, potentially because STIM2 is not expressed at significant levels in immature human T cells to compensate for a lack of STIM1 in contrast to mouse T cells. That said, STIM2 protein levels in naive mouse T cells were below the detection limit in our experiments, and it remains to be addressed whether STIM2 is functional in immature mouse T cells. Treg cell development in *Orai1*-deficient mice is normal despite impaired SOCE in T cells (106, Stefan Feske, unpublished data), and it can be speculated that residual SOCE in *Orai1*-deficient immature T cells in the thymus (potentially mediated by ORAI2 or ORAI3) is sufficient for Treg development.

Impaired development of functional Treg cells in *Stim1/Stim2*-deficient mice and *STIM1*-deficient patients results in an autoimmune, lympho-myeloproliferative phenotype characterized by hepatosplenomegaly and lymphadenopathy (72, 75). In mice, this phenotype can be prevented by transfer of wildtype Treg cells into 2-week-old *Stim1/Stim2*-deficient mice, indicating that the lympho-myeloproliferative disease is mainly caused by the regulatory T-cell defect. In addition, ORAI1-deficient human patients and mice that have normal numbers of Treg cells lacked overt signs of autoimmunity and lymphoproliferation (78, 106). Disease manifestations due to Treg cell deficiency vary between human patients and mice. In *STIM1*-deficient patients, autoimmunity presents as autoimmune hemolytic anemia and thrombocytopenia that was not observed in mice. Conversely, leukocytic infiltration of liver and lung, dermatitis, blepharitis, and colitis were not detected in the patients (72). Taken together, the mouse models for ORAI1 and STIM1/STIM2 function in combination with the characterization of ORAI1- and STIM1-deficient patients have greatly enhanced our understanding of Ca^{2+} signaling and SOCE in immune function *in vivo* and yielded some unexpected results regarding the role of SOCE in T-cell development.

The role of Ca^{2+} influx in immunodeficiency and autoimmune and inflammatory diseases

Immunodeficiency

The importance of SOCE for adaptive immune responses is unequivocal given the severe immunodeficiency observed in patients with mutations in STIM1 and ORAI1 (75, 78) and the functional defects in T cells from *Orai1*-, *Stim1*-, and *Stim2*-deficient mice (72, 106). Aside from T cells, functional defects in other cell types of the adaptive and innate immune system cannot be ruled out to contribute to immunodeficiency in the patients. This assumption is plausible as STIM1 and ORAI1 are expressed in practically all lymphoid and myeloid cells and a role of STIM1 and ORAI1 in SOCE has been demonstrated in B cells (135), macrophages (35), and mast cells (38, 39). In addition, defects in Ca^{2+} signaling unrelated to STIM1 and ORAI1 are likely to contribute to the pathology of other forms of immunodeficiency (reviewed in 2). Ca^{2+} influx was found to be impaired in T and B cells of patients suffering from X-linked agammaglobulinemia (XLA), common variable immunodeficiency (CVID), and Wiskott–Aldrich syndrome (WAS) due to mutations in

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Bruton's tyrosine kinase (*BTK*) (147, 148), *CD19* (149), and WAS protein (WASP), respectively (149, reviewed in 2). CD19 functions by amplifying BCR signaling including Ca^{2+} influx and loss of CD19 function in human patients and mice results in defective Ca^{2+} influx, Ig class switching, and reduced numbers of memory B cells (149, 151, 152). WASP, apart from being a key regulator of F-actin polymerization, has a role in signal transduction in T cells and is involved in, for instance, the regulation of transcription factors NFAT, Erk, Elk1, and c-fos, and IL-2 production (153). While WASP controls Ca^{2+} signaling most likely by acting upstream of store depletion, its homologue WAVE2 (WASP-family verprolin homologous protein 2) has been proposed to directly regulate CRAC channel activation (154). Thus, mutations in a number of genes impair efficient activation of Ca^{2+} influx in lymphocytes and contribute to the severity of immunodeficiency disease.

Autoimmune disease and inflammation

Abnormal lymphocyte Ca^{2+} signaling was observed in several models of autoimmune disease including those for systemic lupus erythematosus (SLE) (2). Removing negative regulators of BCR signaling, thereby modulating the strength of the BCR Ca^{2+} signal, resulted in the occurrence of autoimmunity in a number of transgenic mouse models. Lack of FcγRIIB (155), SH2 domain-containing phosphatase 1 (SHP1) (156), LYN (157), or CD22 (158, 159) is associated with autoantibody production or autoimmune disease in mice. Mature follicular B cells but not transitional stage 1 B cells from $lyn^{-/-}$ and $cd22^{-/-}$ mice showed substantially enhanced Ca^{2+} influx, suggesting that the CD22–LYN–SHP1 pathway attenuates Ca^{2+} responses and B-cell activation in mature B cells and thereby maintains B-cell tolerance (160). Furthermore, Duty *et al.* (161) observed a subset of autoantibody-producing IgD^+ mature B cells that had greatly reduced Ca^{2+} responses, a finding that was interpreted to indicate that these cells are kept in an anergic state to prevent autoreactivity. It should be emphasized, though, that while these observations are intriguing, the evidence is mostly circumstantial, because Ca^{2+} influx is likely to be one of several signaling pathways modulated in the studies described above. In addition, it is difficult to prove that altered Ca^{2+} signaling in T and B cells in fact causes autoimmune disease. Conversely however, inhibition of Ca^{2+} influx has been reported to have an attenuating effect on autoimmunity and inflammation in several animal models. Pharmacological inhibition of K^+ channels expressed in T cells significantly ameliorated disease outcome in animal models of multiple sclerosis (25, 162, 163). This effect is indirect, because in the absence of K^+ channel function, the negative plasma membrane potential required for passive Ca^{2+} influx is dissolved. Inhibition of either of the two K^+ channels expressed in T cells, the voltage-gated potassium channel Kv1.3 (KCNA3) and the intermediate conductance calcium-activated potassium channel IKCa1 (KCNN4), impairs SOCE. As the main effect of K^+ channel inhibition is the attenuation of Ca^{2+} influx, it can be surmised that Ca^{2+} signals are necessary for the autoreactive functions of T cells.

T cells, mast cells, and macrophages are essential mediators of pathophysiological processes in a number of inflammatory diseases including asthma, atherosclerosis, and colitis. Inhibition of Ca^{2+} influx in these proinflammatory cells has been shown to ameliorate disease in a number of animal models. In atherosclerosis, T cells infiltrate atherosclerotic plaques, where they are activated by plasmacytoid dendritic cells resulting in activation of

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macrophages and killing of endothelial cells. Inhibition of IKCa1 K^+ channels in T cells, macrophages, and vascular smooth muscle cells was able to suppress the development of atherosclerosis in the Apoe$^{-/-}$ mouse model (164). The non-store operated Ca^{2+} channel, transient receptor potential family member M2 (TRPM2) was recently shown to be involved in monocyte Ca^{2+} signaling, chemokine production, and activation of inflammatory neutrophils in a mouse model of colitis in which mucosal neutrophil infiltration and ulceration were attenuated in *Trpm2*$^{-/-}$ mice (165). Asthma is an inflammatory immune disease in which mast cells and T cells play a central role in disease pathology (reviewed in 166, 167), and it seems likely that SOCE mediated by STIM1 and ORAI1 is required for activation of both cell types during disease initiation and progression (38, 39, 72, 106). Mast cells and T cells from *Stim1*- and *Orai1*-deficient mice were severely impaired in cytokine production (38, 39, 72, 106), and mast cells in addition showed defects in degranulation *in vitro* upon FcεRI stimulation and anaphylactic responses *in vivo* (38, 39). Evidence for an involvement of Ca^{2+} influx in asthma also comes from *Slat*-deficient mice that lack Ca^{2+} store depletion and influx and that are impaired in their ability to mount both Th1 and Th2 inflammatory responses in their lungs (168). More roles for SOCE, STIM1, and ORAI1 in autoimmune and inflammatory diseases are likely to be discovered in the future.

Conclusion

With the molecular identification of STIM1 and ORAI1, the mechanisms and role of SOCE and CRAC channel function in cells of the immune system and other tissues are far better understood than just a few years ago. *In vitro* experiments showed that ORAI1 and STIM1 are sufficient to mediate SOCE through CRAC channels, and many details regarding the structure, functional domains, and interactions of STIM1 with ORAI1 have emerged. The details of how ORAI1 functions as the CRAC channel and how it is gated by STIM1 need to be elucidated. Significant insight can be expected from solving the crystal structure of both STIM1 and ORAI1. Most research has focused on ORAI1 and STIM1 both *in vitro* and *in vivo*, where several transgenic mouse models for *Stim1* and *Orai1* have been generated. In addition, the identification of patients with hypomorphic mutations in ORAI1 and STIM1 has illustrated that both genes are indispensable for lymphocyte function, skeletal muscle function, and dental enamel formation. The function and *in vivo* role of the paralogues ORAI2, ORAI3, and STIM2 remain largely unresolved, although they seem to have overlapping functions with ORAI1 and STIM1 *in vitro*. All five molecules are expressed in a large variety of tissues, and it needs to be determined if different combinations of STIM and ORAI proteins mediate SOCE in diverse tissues or if STIM1 and ORAI1 are the main regulators of SOCE. An exclusive role for STIM1 and ORAI1 is unlikely given the observation of SOCE in many cell types and the limited spectrum of disease in patients lacking functional ORAI1 and STIM1 comprising 'only' immunodeficiency, ectodermal dysplasia, and myopathy.

In the immune system, SOCE and expression of STIM1 and ORAI1 have been observed in many cell types involved in innate and adaptive immune responses. Lack of STIM1 or ORAI1 in human patients and mice strongly compromises T-cell function, but data from mouse models indicate that mast cell, phagocyte, and B-cell function are also impaired in the absence of SOCE. Nevertheless, the role of SOCE, ORAI1, and STIM1 in adaptive and

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innate immune responses is not well understood yet. While functional responses of immune cells are impaired their development from hematopoietic stem cells seems unperturbed, which is especially puzzling for T cells because Ca^{2+} signals have been implicated in T-cell development and thymic selection. Other Ca^{2+} channels need to be considered and the role of Ca^{2+} released from ER stores revisited during T-cell development. By contrast, lack of SOCE interferes with the development of regulatory T cells both in human patients and mice, although the mechanisms underlying this specific Ca^{2+} requirement for Treg cell differentiation remain unclear. Similarly, it is not known how SOCE is regulated in different types of T cells such as Th1, Th2, Th17, and Treg cells and whether altering SOCE in these subsets is involved in shifting the balance between normal immune responses and autoimmunity and inflammation. Given the strong attenuation of T-cell and mast cell function in the absence of STIM1 and ORAI1, it is not unreasonable to expect that inhibition of SOCE will have beneficial therapeutic effects in inflammatory and autoimmune diseases.

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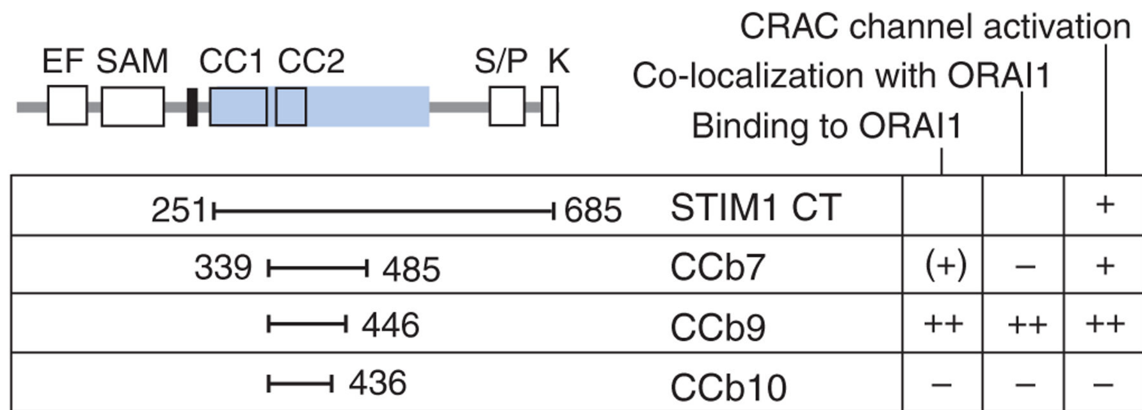

Construct		Binding to ORAI1	Co-localization with ORAI1	CRAC channel activation
251–685	STIM1 CT			+
339–485	CCb7	(+)	–	+
–446	CCb9	++	++	++
–436	CCb10	–	–	–

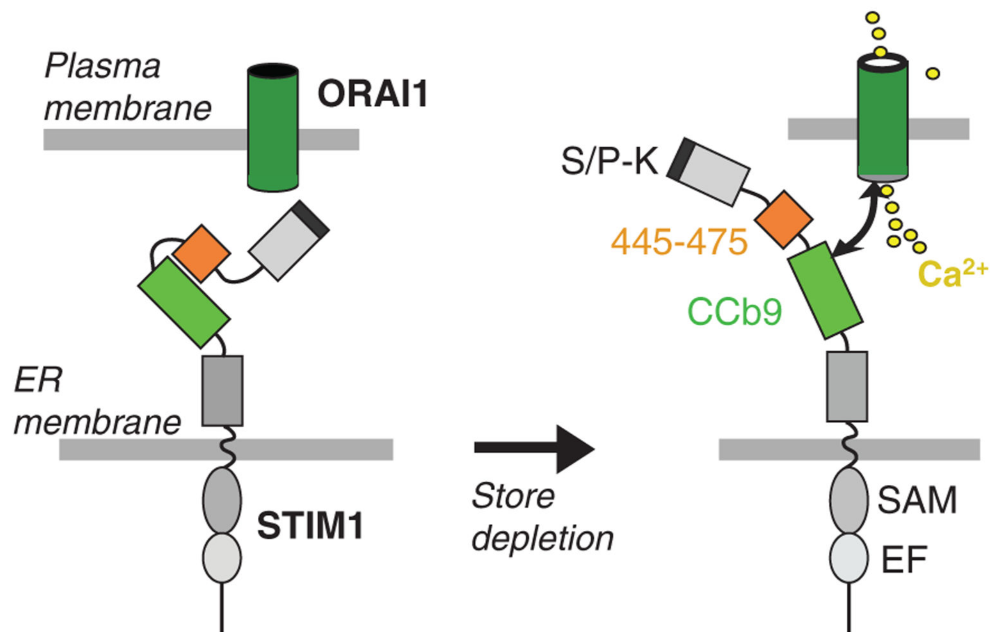

Fig. 1. A minimal ORAI1 activation domain in STIM1 (CCb9).

In the resting state with replete Ca^{2+} stores, the CCb9 ORAI1-activation domain in STIM1 is masked by an inhibitory $STIM1_{445–475}$ peptide. Upon store depletion, the CCb9 domain is released, binds to and activates the ORAI1–CRAC channel. CC, coiled-coil; CT, STIM1 C-terminus; EF, EF hand; ERM, ezrin⁄radixin⁄moesin; SAM, sterile α motif; TM, transmembrane; S⁄P, serine–proline; K, lysine. Modified and reproduced with permission from (66).

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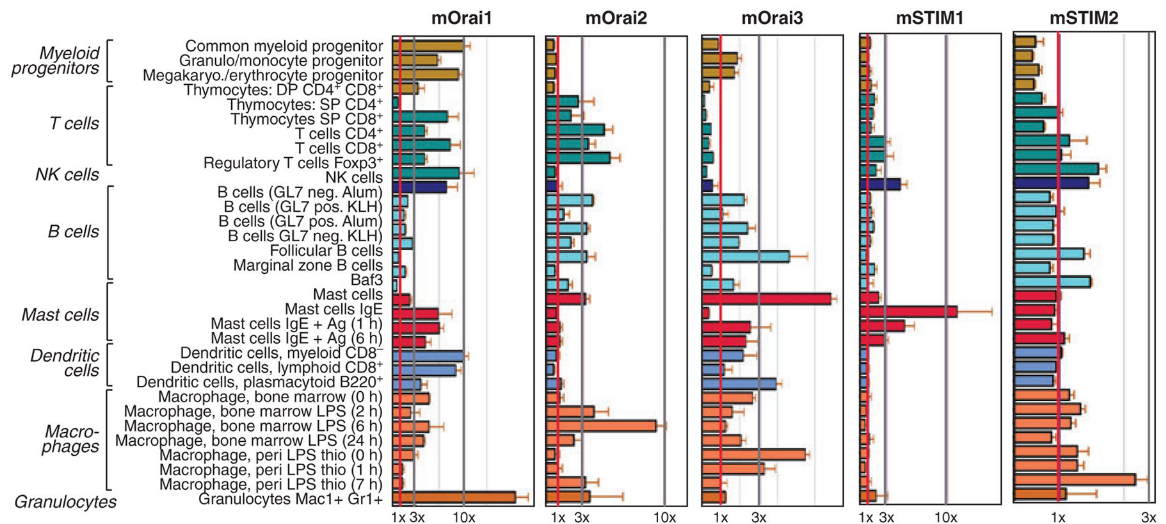

Fig. 2. Murine Orai and Stim family members are expressed in a wide variety of cells in the immune system.

mRNA levels of mStim and mOrai based on expression data retrieved from BioGPS (http://biogps.gnf.org). Cell lines and tissues were sourced from 8–10-week-old C57Bl/6 mice and mRNA expression analyzed using Affymetrix MOE430_2 microarrays (102). Vertical gray lines indicate 1×, 3×, and 10× fold expression over the median of all tissues analyzed.

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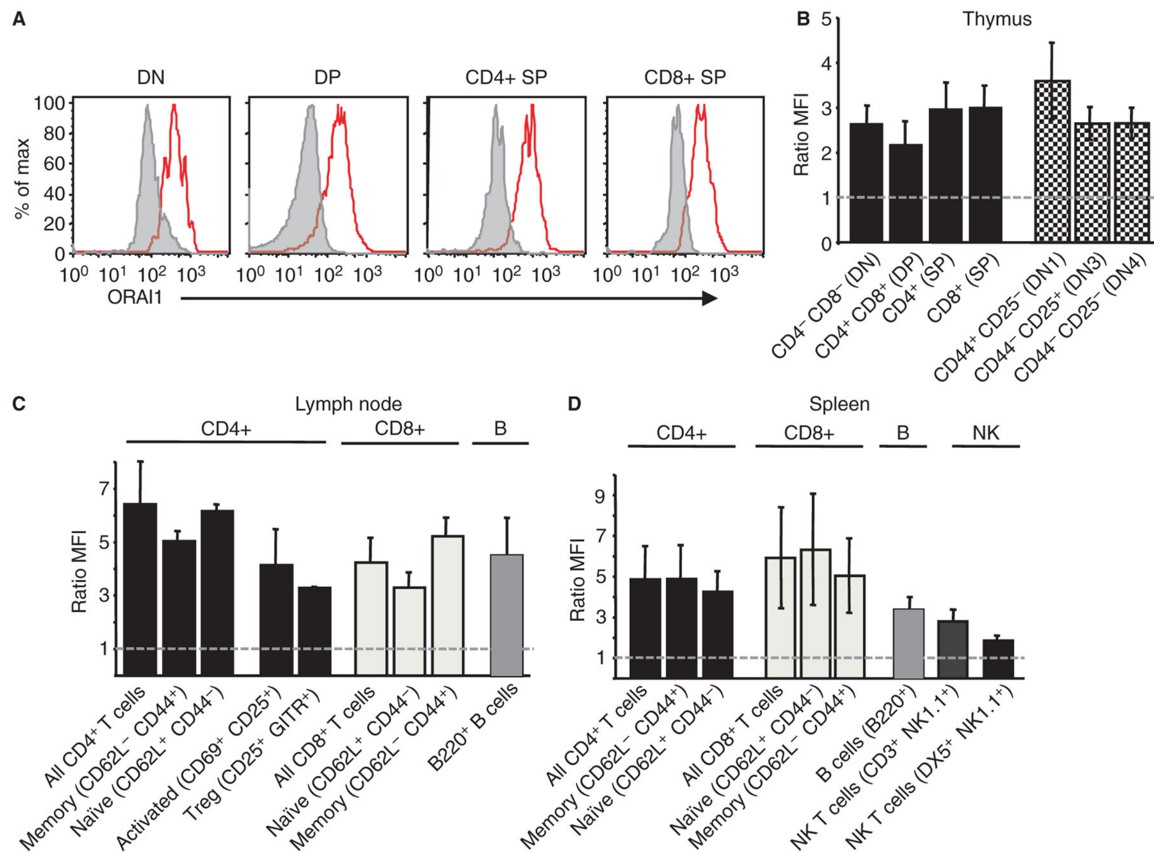

Fig. 3. ORAI1 expression in developing and mature lymphocytes.

ORAI1 protein expression was analyzed in cells isolated from primary and secondary lymphoid organs of 6–8-week-old C57Bl/6 mice incubated with polyclonal anti-ORAI1 antibody or secondary antibody alone. (A,B) ORAI1 is expressed at all stages of T-cell development in the thymus. (C,D) ORAI1 expression in T, B, and NK cells in lymph node and spleen as indicated. ORAI1 expression was comparable in lymphocyte populations at various stages of differentiation and activation; weakest expression was found in splenic NK cells ($n = 8$) and highest expression in lymph node $CD4^+$ T cells. Expression is shown as the ratio of MFI ORAI1 (red trace in A): MFI secondary antibody alone (gray trace in A). $n = 2–8$ mice analyzed (spleen), $n = 2–19$ (lymph node), $n = 6–10$ (thymus). MFI, mean fluorescence intensity.

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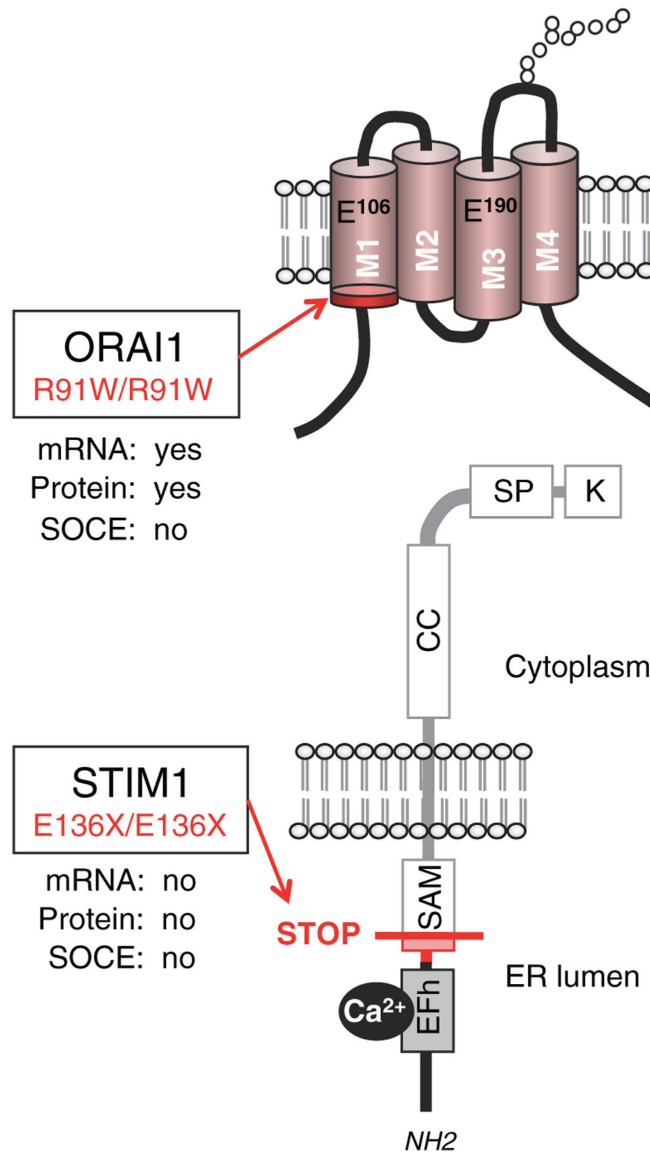

Fig. 4. Mutations in *ORAI1* and *STIM1* in immunodeficient patients lacking store-operated Ca^{2+} influx.

Locations of homozygous missense and nonsense mutations in ORAI1 and STIM1. An arginine to tryptophan single amino acid substitution at the cytoplasmic end of the first transmembrane domain of ORAI1 results in expression of a nonfunctional protein ($ORAI1^{R91W}$) (77). An insertion mutation in codon 128 of STIM1 results in a frame shift (fs) and premature STOP (X) in codon 136; the frameshifted region of STIM1 protein is indicated in red. No STIM1 mRNA or protein is found in cells of the patient.

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Table 1.

Clinical and immunological phenotypes of ORAI1- and STIM1-deficient patients

	ORAI1	**STIM1**
Gene defect	R91W/R91W	E136X/E136X
Chromosome and inheritance	12q24 homozygous (AR)	11p15 homozygous (AR)
No of Patients	Two (P1, P2)	Three (P3–P5)
Gene expression & Ca^{2+} channel function		
mRNA/protein	Present/present	Absent/absent
SOCE/I_{CRAC}	Absent/absent	Absent/not tested
Cell types tested	T cells, B cells, fibroblasts	Fibroblasts
Immunological manifestations		
Immunodeficiency & infections	P1: BCG infection, rota virus enteritis, Interstitial pneumonia, gastrointestinal sepsis P2: none; HSCT at 4 m	P3: Pneumonia, urinary tract infections, sepsis (*Escherichia coli, Streptococcus pneumoniae*); cytomegalo and varizella zoster virus P4: Epstein Barr virus, viral enteritis, enteroviral encephalitis P5: sepsis; HSCT at 15 m
Lymphocyte counts	Normal	Normal
Lymphocyte subsets	Normal	$CD4^+$ $CD25^+$ $Foxp3^+$ Treg ↓
T cell activation (*in vitro*)	Proliferation ↓↓ Cytokine production ↓↓	Proliferation ↓-↓↓
Antibody production	Normal - ↑ Ig levels (infections), no specific Ab response	Normal Ig levels, no specific Ab response
Autoimmunity & lymphoproliferation	No	AIHA (P3, P4), Thrombocytopenia (P3–P5), splenomegaly & lymphadenopathy (P3, P4)
Extraimmunological manifestations		
Congenital myopathy	Yes	Yes
Ectodermal dysplasia	Enamel dentition defect (Hypocalcified amelogenesis imperfecta) Anhydrosis	Enamel dentition defect
Other	No	P4: Nephrotic syndrome
Outcome	P1: Death from sepsis (11 m) P2: Survival after HSCT (now 15 y)	P3: Death from HSCT complications (9 y) P4: Death from encephalitis (18 m) P5: Survival after HSCT (now 6 y)
References	78,107,110,115,116	75

Patients lacking STIM1 and functional ORAI1 suffered from similar clinical syndromes comprising immunodeficiency early in life, congenital myopathy, and an enamel calcification defect. STIM1-deficient patients in addition presented with autoimmunity and lymphoproliferation. Ab, antibody; AIHA, autoimmune hemolytic anemia; AR, autosomal recessive; BCG, Bacille Calmette-Guérin; CMV, cytomegalovirus; I_{CRAC}, Ca^{2+} release-activated Ca^{2+} (CRAC) channel current; HSCT, hematopoietic stem cell transplantation; m, months; P, patient; SOCE, store-operated Ca^{2+} entry; y, years.

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Table 2.

Summary of main clinical findings associated with CRAC channelopathy due to mutations in human *STIM1* and *ORAI1*

	ORAI1	STIM1
Immunodeficiency		
Viral, bacterial, fungal infections	+	+
Autoimmunity		+
Autoimmune hemolytic anemia		+
Thrombocytopenia		+
Lymphoadenopathy/ hepatosplenomegaly		+
Congenital myopathy	+	+
Ectodermal dysplasia		
Enamel dentition defect	+	+
Anhydrosis	+	

For details see Table 1.

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Table 3.

Phenotypes of transgenic mice lacking *Stim1, Stim2*, and *Orai1* gene expression

	Orai1	Stim1	Stim2	Stim1/Stim2
Gene-targeting method	Deletion (38,106,127); knock-in (129)	Deletion (all)	Deletion	Deletion
	gt(38, 127); hr (106,129)	gt (35,103,126,136); hr (39,72)	hr	hr
	Conventional (all)	Conventional (35,103,126,136) loxP: CMV-Cre (39,72); CD4-Cre (72)	loxP: CD4-Cre, CMV-Cre	loxP: CD4-Cre, CMV-Cre
Viability	Perinatal death & runted (all)	Perinatal death & runted: conventional (35,103,126,136), CMV-Cre (39,72) Viable: CD4-Cre (72)	Death 4–5 w p.p.: CMV-Cre Viable: CD4-Cre	Viable: CD4-Cre
Ca^{2+} influx	Mast cells: ↓↓ (38) T cells: normal(38), ↓↓ (106) B cells: ↓↓ (106) Platelets: ↓↓ (127,129)	Mast cells: ↓↓ (39) T cells: ↓↓ (72,136) Macrophages: ↓↓ (35) Platelets: ↓↓ (126) Skeletal myotubes: ↓↓ (103)	T cells: peak normal, sustained $[Ca^{2}+]i$ ↓	T cells: ↓↓ $Foxp3^+$ Treg: ↓↓ MEF: ↓↓
Immunological phenotypes				
In vitro	Mast cells: degranulation ↓↓: LTC_4 synthesis ↓↓: cytokines ↓ (38) T cells: cytokines ↓ (38) to ↓↓ (106) B cells: proliferation ↓ (106)	Mast cells: degranulation ↓, cytokines ↓ (39) T cells: cytokines ↓↓ (72) Macrophage function ↓ (35)	T cells: cytokines ↓	T cells: cytokines ↓↓
In vivo	Anaphylaxis *in vivo* ↓ (38)	Anaphylaxis *in vivo* ↓ (39) T-depend. B cell reponse normal (136) Ability of T cells to induce GvHD normal to ↓ (136) Protection from autoimm. hemolytic anemia and thrombocytopenia (35)		Autoimmunity (lympho-myeloproliferation)
Development	Normal: T cells (38,106), $Foxp3^+$ T_{reg} (106), B cells (106), Mast cells (38)	Normal: T cells (72,136), $Foxp3^+$ Treg (72,136), Mast cells (39)	Normal: T cells	Normal: T cells, B cells Treg ($Foxp3^+$) ↓↓
Non-immunological Phenotypes				
	Myopathy (38,106) Hair loss (106) Platelet activation ↓↓ (127,129), protection from thromboischemia (127)	Myopathy (103) Platelet activation ↓↓ (126), protection from thromboischemia (126)		
References	(38, 106, 127, 129)	(35, 39, 72, 103, 126, 136)	(72)	(72)

This table provides a summary of mouse lines generated by homologous recombination (hr) using conventional or conditional (loxP/Cre) gene-targeting and insertional mutagenesis (gt, gene-trapping), respectively. All *Orai1*- and *Stim1*-deficient mice displayed early postnatal lethality and a runted phenotype. CMV, cytomegalovirus; GvHD, graft-versus-host disease; LTC_4, leukotriene C4; MEF, mouse embryonic fibroblasts; p.p., post partum; w, week.