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ORAI1 Deficiency Impairs Activated T Cell Death and Enhances T Cell Survival

Kyun-Do Kim,* Sonal Srikanth,* Ma-Khin Win Yee,* Dennis C. Mock,† Gregory W. Lawson,‡ and Yousang Gwack*

ORAI1 is a pore subunit of Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels that mediate TCR stimulation-induced Ca\(^{2+}\) entry. A point mutation in \textit{ORAI1} (\textit{ORAI1}\(^{R89T}\)) causes SCID in human patients that is recapitulated in \textit{Orai1}\(^{-/-}\) mice, emphasizing its important role in the immune cells. In this study, we have characterized a novel function of ORAI1 in T cell death. CD4\(^{+}\) T cells from \textit{Orai1}\(^{-/-}\) mice showed robust proliferation with repetitive stimulations and strong resistance to stimulation-induced cell death due to reduced mitochondrial Ca\(^{2+}\) uptake and altered gene expression of proapoptotic and antiapoptotic molecules (e.g., Fas ligand, Noxa, and Mcl-1). Nuclear accumulation of NFAT was severely reduced in ORAI1-deficient T cells, and expression of ORAI1 and a constitutively active mutant of NFAT recovered cell death. These results indicate NFAT-mediated cell death pathway as one of the major downstream targets of ORAI1-induced Ca\(^{2+}\) entry. By expressing various mutants of ORAI1 in wild-type and \textit{Orai1}\(^{null}\) mice, we have shown that activation-induced cell death is directly proportional to the intracellular Ca\(^{2+}\) concentration levels. Consistent with the in vitro results, \textit{Orai1}\(^{-/-}\) mice showed strong resistance to T cell death induced by injection of anti-CD3 Ab. Furthermore, ORAI1-deficient T cells showed enhanced survival after adoptive transfer into immunocompromised hosts. Thus, our results demonstrate a crucial role of the ORAI1–NFAT pathway in T cell death and highlight the important role of ORAI1 as a major route of Ca\(^{2+}\) entry during activated T cell death. \textit{The Journal of Immunology}, 2011, 187: 000–000.

stimulation of TCR evokes Ca\(^{2+}\) entry via Ca\(^{2+}\)-release-activated Ca\(^{2+}\) (CRAC) channels (1, 2). An increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) induces proliferation and cytokine production of immune cells by activation of downstream target molecules including calmodulin (CaM), calcineurin, and the transcription factor NFAT (2). The Ca\(^{2+}\)-activated CaM/calcineurin protein phosphatase complex dephosphorylates heavily phosphorylated, cytoplasmic NFAT, which in turn translocates into the nucleus and turns on various transcriptional programs. Recently, ORAI1 (CRACM1) was identified as a pore component of CRAC channels by genome-wide RNA interference high-throughput screens (3–6). It was also shown that human patients with a homozygous missense mutation in \textit{ORAI1} suffer from lethal SCID (5). Another important signaling molecule in the CRAC channel pathway, stromal interaction molecule 1 (STIM1), was identified earlier using limited RNA interference screens in \textit{Drosophila} and mammalian cells (7, 8). Stimulation of the TCR activates phospholipase \(\mathrm{C}_{\gamma}\), which cleaves phospha-diylinositol 4,5-bisphosphate to produce the second messenger inositol 1,4,5-trisphosphate, which in turn binds to inositol 1,4,5-trisphosphate receptors on the endoplasmic reticulum (ER) membrane and depletes the ER Ca\(^{2+}\) stores. STIM1 senses Ca\(^{2+}\) depletion via its EF-hands and translocates into the plasma membrane-proximal regions to activate ORAI1, thereby causing a sustained increase in [Ca\(^{2+}\)]\(_{i}\) (7, 9, 10). This sequential activation mechanism was termed store-operated Ca\(^{2+}\) entry (SOCE) because depletion of ER Ca\(^{2+}\) stores precedes CRAC channel activation (11). Recently, three siblings from one kindred have been identified with homozygous nonsense mutation in \textit{STIM1}. These patients also suffered from SCID, further emphasizing the crucial role of CRAC channels in the immune system (12).

Recently, several reports have described the immune phenotypes of ORAI1- and STIM1-deficient mice (13–18). These mice showed a defect in immune cells consistent with the SCID patients. CD4\(^{+}\), CD8\(^{+}\) effector T cells, and mast cells from ORAI1-deficient mice showed a decrease in SOCE as well as cytokine production (13, 14). In addition, ORAI1 deficiency impaired Ca\(^{2+}\) influx and effector functions of neutrophils as well as platelets (15, 16). STIM1 deficiency also showed a pronounced decrease in SOCE and cytokine production in T cells, resulting in resistance to experimental autoimmune encephalomyelitis (EAE) (17, 18). On the contrary, mice deficient in STIM2, another member of the STIM family, showed a mild defect in SOCE and correspondingly succumbed to EAE, albeit with less severe symptoms (17). Mice lacking both STIM1 and STIM2 displayed lymphoproliferative disorders in addition to SCID phenotype and were completely resistant to EAE (18, 19). Their lymphoproliferative phenotype was attributed to a severe reduction in regulatory T cell population (18). Interestingly, SCID patients harboring mutations in \textit{STIM1} also showed enlarged lymph nodes and elevated memory T cell populations (12).
TCR signaling plays an important role in immune homeostasis for maintenance of T cell numbers and induction of cell death. Cell death induced by TCR stimulation is critical for homeostasis of peripheral T cells after Ag clearance and negative selection of autoreactive T cells in the thymus (20–22). Activated T cell death occurs through two major apoptotic pathways, the death receptor- and mitochondria-mediated pathways. Death receptor-mediated apoptosis involves the Fas ligand (FasL)/Fas signaling pathway majorly regulated by NFAT (23, 24), whereas mitochondria-mediated cell death occurs due to loss of mitochondrial membrane potential (20). Mitochondria-mediated cell death pathway involving the Bcl-2 family members (e.g., Bcl-2 and Bcl-XL) and the BH3-only proteins (e.g., Bad, Bik, Bim, and Noxa) plays an important role in T cell death and survival, as seen in isolated T cells and in animal models (20, 22, 25). Double-knockout (KO) mice lacking expression of ORAI1 and Bim show severe lymphoproliferative disorders and marked resistance to cell death, indicating an important role of both death receptors and mitochondria in T cell death (26–28).

Earlier, it was noticed that T cell death mediated by increased [Ca2+]i, upon TCR stimulation can be mimicked by treatment with the ionophore ionomycin (29). In cell death induced by TCR stimulation, the relation between Ca2+ homeostasis and Bcl-2 family members such as Bax, Bak, Bcl-2, and Bcl-XL has been extensively studied (30–33). These studies indicate that ER Ca2+ homeostasis is important for T cell death by modulation of cytosolic free Ca2+, mitochondrial Ca2+ uptake, or Ca2+ entry. A relationship between Ca2+ entry and mitochondrial Ca2+ uptake in T cells has been implicated in numerous studies. T cells have been shown to accumulate Ca2+ in mitochondria upon elevation of [Ca2+]i, and conversely, mitochondrial Ca2+ buffering is important for prolonged CRAC channel activity, NFAT activation, and induction of cell death (34–37). Furthermore, it was shown that in T cells, mitochondria actively translocate toward the immunological synapse, accumulate Ca2+, and prevent Ca2+ dependent inactivation of CRAC channels (38, 39). Although in vitro pharmacological studies suggest an important role of Ca2+ in cell death after TCR stimulation, the exact role of Orai1 in mitochondrial Ca2+ uptake and T cell death has not been investigated due to lack of an appropriate animal model. It is also puzzling how the same Ca2+ signaling pathway can play a critical role in various outcomes of proliferation, death, and tolerance of T cells. If the amplitude or frequency of Ca2+ entry governs the fate of T cells as proposed previously (40–42), the threshold levels of [Ca2+]i for such decisions need to be determined.

In this study, we investigated how different levels of Ca2+ entry influence death and survival of T cells in vitro and in vivo using Orai1−/− mice. We showed that Orai1-deficient T cells are strongly resistant to cell death due to reduction in death receptor- and mitochondria-mediated cell death mechanisms. Based on the results from recovery experiments by expression of Orai1 and constitutively active (CA)-NFAT in Orai1−/− T cells, we determined a crucial role of the Orai1–NFAT pathway in T cell death. Using Orai1-deficient cells and expression of a dominant-negative mutant of Orai1, we investigated how diverse levels of Ca2+ entry can influence T cell death. In support of the results from isolated T cells, Orai1−/− mice showed strong resistance to depletion of T cells upon injection of anti-CD3 Ab in vivo. In addition, survival of Orai1-deficient T cells was enhanced after transfer into immunocompromised hosts. To our knowledge, our results suggest for the first time a strong correlation between elevated [Ca2+]i, via Orai1 and T cell death/survival using a genetically manipulated animal model.
Technology), anti-Bax (clone D2E11; Cell Signaling Technology), anti-NEAT1 (purified rabbit polyclonal Ab to the 67.1 peptide of NEAT1; a kind gift from Dr. Anjana Rao), and anti-Mcl-1 (Rockland). Chemiluminescence images were acquired using an Image Reader LAS-3000 LCD camera (Fujifilm). Band intensities were quantified using Multi Gauge V 3.0 software (Fujifilm).

Surface staining, measurement of mitochondrial membrane potential, and cell cycle analyses
All Abs used for flow cytometry were purchased from eBioscience, and staining was performed according to the manufacturer’s instructions. The following Abs were used for surface staining: anti-CD4 (FITC, clone GK1.5), anti-CD8 (PE, clone 53-6.7), and anti-CD2 (PE, clone 2H7). Anti-CD3 (PE, clone 145-2C11) was used for the intracellular staining. For intracellular staining, CD4+ T cells were cultured in nonpolarizing conditions until day 5 and then stained with 1 μg/ml plate-coated anti-CD3 Ab for 24 h, and then mitochondrial mass was measured by incubating cells with 50 nM MitoTracker Deep Red (Invitrogen) for 15 min at 37°C. For cell-cycle analysis, cells were cultured with 1 μg/ml anti-CD3 Ab and 1 μg/ml anti-CD28 Ab (BD Pharmingen) for 24 or 48 h in a plate coated with 0.3 mg/ml goat-anti-hamster, followed by incubation in a solution containing propidium iodide (50 nM). Cell death was determined by PI staining. T cells were considered to have nuclear NFAT1 when >80% of the cells exhibited nuclear translocation. Nuclear translocation was assessed by counting the number of PI-staining nuclei and quantified using the ImageXpress automated imaging system and analyzed by MetaXpress software (Molecular Devices). Nuclear translocation was assessed by calculation of a correlation of the intensity of NFAT1 staining and DAPI staining. T cells were considered to have nuclear NFAT1 when >80% of the FITC-anti-NFAT1 staining coincided with the fluorescence signal from DAPI. Each data point represents an average of at least 450 individual cells per well.

Statistical analysis
Statistical analysis was carried out using the two-tailed Student t test. Differences were considered significant when p values were <0.05.

Results
ORAII-deficient CD4+ effector T cells show strong resistance to TCR stimulation-induced cell death
To determine how reduced SOCE by ORAII deficiency influences T cell proliferation, first we examined the number of ORAII+/+ and ORAII−/− naive and effector CD4+ T cells after stimulation with plate-coated anti-CD3 and anti-CD28 Abs. Six days after stimulation, the number of ORAII−/− T cells did not show a significant difference with that of WT T cells (Fig. 1A, 1st stimulation). However, after restimulation, the number of ORAII−/− effector T cells showed a robust increase, whereas that of WTeffector T cells increased modestly (Fig. 1A, 2nd stimulation). Furthermore, ORAII-deficient T cells continuously responded to repetitive stimulations and expanded robustly, whereas WT T cells disappeared, possibly by undergoing cell death (Fig. 1A). ORAII−/− T cells were capable of expanding up to at least 12 stimulations that we have tested (data not shown). Because the number of T cells is the summation of cell death and proliferation rate, we measured both the rate of proliferation and cell death in ORAII+/+ and ORAII−/− effector T cells. The measurements of the CFSE dilution rate together with cell cycle analysis showed a slightly enhanced proliferation rate and cell cycle progression of ORAII−/− T cells, especially at the early stages of 48 and 72 h poststimulation (Fig. 1B, Supplemental Fig. 1A). Surprisingly, we observed a 2-fold increase in ORAII−/− live cell populations as determined by side and forward scatter after stimulation, suggesting that ORAII−/− T cells may exhibit resistance to cell death (Supplemental Fig. 1B). To directly measure cell death, we differentiated naive CD4+ T cells to effector cells and treated them with various concentrations of plate-coated anti-CD3 Ab for 6 h. ORAII−/− effector T cells showed a marked decrease in Annexin V+/− populations as compared with WT cells at all the concentrations of anti-CD3 Ab tested (Fig. 1C). As previously reported for human T cells lacking functional ORAII (5), ORAII-deficient T cells showed a marked decrease in SOCE depending on gene dosage, with heterozygous cells showing intermediate levels of SOCE when compared with ORAII+/+ and ORAII−/− cells (Supplemental Fig. 2A, 2B).
In addition to regulation of peripheral T cell homeostasis, Ca\(^{2+}\) signaling and TCR stimulation-mediated cell death play a major role during negative selection in the thymus (42, 46–48). To measure cell death of thymocytes, we stimulated \(\text{Orai}^{1+/+}\) and \(\text{Orai}^{1-/-}\) thymocytes with PMA and ionomycin (Fig. 2A). Ionomycin or anti-CD3 Ab alone did not induce robust cell death in thymocytes (data not shown), consistent with previous reports that costimulation is also required for negative selection (47). The live cell populations determined by forward and side scatter were significantly higher in stimulated \(\text{Orai}^{1-/-}\) thymocytes (Fig. 2A). In addition, the apoptotic cell population of \(\text{Orai}^{1-/-}\) thymocytes as judged by Annexin V\(^+\) or 7-AAD\(^+\) cells was markedly reduced after stimulation (Fig. 2B). Previously, a very marginal difference in single positive (SP) CD4\(^+\) and CD8\(^+\) T cell populations was observed in young, 6–8-wk-old \(\text{Orai}^{1+/-}\) and \(\text{STIM}1^{+/-}\) mice (13, 14, 49). Consistent with these results, we observed a minor difference in the thymi of 6–8-wk-old \(\text{Orai}^{1+/-}\) and \(\text{Orai}^{1-/-}\) mice (Fig. 2C). However, significantly higher populations of CD4\(^+\) and CD8\(^+\) SP cells and a lower percent of double-positive cells appeared in the thymi of \(\text{Orai}^{1-/-}\) mice older than 12 wk (Fig.

**FIGURE 1.** ORAI1-deficient T cells are resistant to cell death induced by stimulation. A, Growth curves of \(\text{Orai}^{1+/-}\) (WT) and \(\text{Orai}^{1-/-}\) (KO) T cells upon repetitive stimulations. Naive CD4\(^+\) T cells purified from 6-wk-old \(\text{Orai}^{1+/-}\) and \(\text{Orai}^{1-/-}\) mice were stimulated with plate-bound anti-CD3 (1 \(\mu\text{g/ml}\)) and soluble anti-CD28 (1 \(\mu\text{g/ml}\)) Abs for 2 d. Five days poststimulation, cells were restimulated with plate-bound anti-CD3 (0.5 \(\mu\text{g/ml}\)) and soluble anti-CD28 (1 \(\mu\text{g/ml}\)) Abs for 24 h, and the cell number was counted daily (2nd stimulation). The same procedure was followed for consecutive 3rd and 4th stimulations. The graphs depict the average number of cells \pm SD from three independent experiments. After the second stimulation, the control WT T cells were not counted because most of them died (indicated as ND). B, Measurement of the proliferation rate of \(\text{Orai}^{1+/-}\) and \(\text{Orai}^{1-/-}\) effector T cells by CFSE labeling. \(\text{Orai}^{1+/-}\) and \(\text{Orai}^{1-/-}\) effector T cells were labeled with CFSE and stimulated with plate-coated anti-CD3 and anti-CD28 Abs. At days 3 and 4 poststimulation, the proliferation rate was examined by flow cytometry. C, Effector T cells were restimulated with various concentrations of anti-CD3 Ab for 6 h, and cell death was assessed by Annexin V and 7-AAD staining. Representative data from five independent experiments are depicted.

(14). Together, these results suggest that the robust increase in the numbers of \(\text{Orai}^{1-/-}\) effector T cells responding to repetitive stimulations is predominantly due to strong resistance to cell death with minor contributions from the increased proliferation rate.

**FIGURE 2.** ORAI1-deficient thymocytes are resistant to cell death induced by TCR stimulation. A, Thymocytes isolated from 6-wk-old \(\text{Orai}^{1+/-}\) (WT) and \(\text{Orai}^{1-/-}\) (KO) mice were stimulated with PMA (20 ng/ml) and ionomycin (1 \(\mu\text{M}\)) for 16 h. Representative flow cytometric forward (FSC) and side (SSC) scatter profiles used to define live-cell populations are depicted. The bar graph on the right represents mean \pm SD of live-cell populations from three independent experiments. B, Live cell gates (depicted in A) were further examined for apoptotic cells using Annexin V and 7-AAD staining. The bar graph on the right shows average \pm SD of 7-AAD and/or Annexin V\(^+\) cell populations from three independent experiments. C, CD4\(^+\) and CD8\(^+\) T cell populations were analyzed from the thymus of 6–8- or 12–16-wk-old \(\text{Orai}^{1+/-}\) and \(\text{Orai}^{1-/-}\) mice. Cells were analyzed by surface staining and flow cytometry. The left panel shows representative data from three independent experiments, whereas the average \pm SD is shown in the bar graph on the right.
2C). These results suggest that Orai1-mediated Ca\(^{2+}\) entry is dispensable for development of T cells in the thymus; however, some of the thymocytes may escape cell death and accumulate with age. Our results in Fig. 2A–C are consistent with earlier observations indicating that Ca\(^{2+}\) entry in thymocytes set up a threshold for negative selection (42, 46, 48), although the defects in Orai\(^{1/-}\) mice are not strong enough to influence T cell development in young mice. We conclude that ORAI1 plays a major role in stimulation-induced cell death of peripheral T cells and also affects, to a lesser extent, cell death of thymocytes.

**ORAI1 deficiency inhibits mitochondria-mediated cell death pathways in effector T cells**

To elucidate the mechanism of strong resistance to cell death in Orai\(^{1/-}\) effector T cells (Fig. 1), we examined functions of major signaling pathways involved in induction of cell death, including mitochondrial Ca\(^{2+}\) uptake and the downstream transcriptional events. Ca\(^{2+}\) accumulates in mitochondria upon elevation of [Ca\(^{2+}\)]\(_{i}\) in T cells, and, reversely, Ca\(^{2+}\) buffering by mitochondria is important for sustained CRAC channel activity, suggesting a reciprocal relationship between CRAC channels and mitochondria (34–37). To examine mitochondrial functions in Orai\(^{1/-}\) T cells upon stimulation, we stained ionomycin or anti-CD3 Ab-stimulated Orai\(^{+/}\) or Orai\(^{1/-}\) T cells with Mitotracker-Deep Red 633, a dye that binds to actively respiring mitochondria. In WT cells, stimulation with ionomycin and anti-CD3 Ab increased the Mitotracker\(^{low}\) cell population, indicative of cells lacking functional mitochondria (Fig. 3A). In contrast, this Mitotracker\(^{low}\) population was substantially reduced in Orai\(^{1/-}\) cells, possibly due to reduced accumulation of Ca\(^{2+}\) in the mitochondria (Fig. 3A).

Previous studies have reported that location of mitochondria close to the site of Ca\(^{2+}\) entry is important to maintain sustained Ca\(^{2+}\) entry via CRAC channels in T cells (38, 39). Interestingly ORAI1 also translocates into the immunological synapse, resulting in localized Ca\(^{2+}\) influx at the immunological synapse (50, 51). Because Orai1 is a critical component of the CRAC channels, these results suggest that Ca\(^{2+}\) entry via Orai1 may serve as a route for mitochondrial Ca\(^{2+}\) uptake in T cells. To investigate this hypothesis, we measured mitochondrial Ca\(^{2+}\) accumulation in Orai\(^{+/}\) and Orai\(^{1/-}\) cells. Although control Orai\(^{+/}\) T cells showed a robust increase in mitochondrial [Ca\(^{2+}\)] upon store depletion, we observed minimal Ca\(^{2+}\) uptake by mitochondria from Orai\(^{1/-}\) T cells (Fig. 3B). Ca\(^{2+}\) ions accumulated in the mitochondria were from the extracellular medium, because we did not observe any mitochondrial Ca\(^{2+}\) accumulation in Ca\(^{2+}\)-free external solutions (Fig. 3B, right panel). These data indicate that in T cells, ORAI1 plays a crucial role in mitochondrial Ca\(^{2+}\) accumulation and thereby in the mitochondria-mediated cell death pathway.

**ORAI1 deficiency influences gene expression patterns of pro- and antiapoptotic genes**

Death receptor-mediated Fas/FasL signaling pathway plays a critical role in T cell death after stimulation (20–22, 52–54), and it has been shown that expression of FasL is regulated by the Ca\(^{2+}\)–NFAT pathway using NFAT-deficient cells and microarray analysis of ionomycin-treated primary T cells (23, 55). In addition, non–death receptor-mediated cell death pathways involving Bcl-2 family members (e.g., Bcl-2 and Bcl-X\(_L\)) and the BH3-only proteins (e.g., Bad, Bik, Bim, and Noxa) are also critical for survival and death of T cells (20, 22, 25). We hypothesized that reduced cell death of Orai\(^{1/-}\) T cells may involve altered gene expression of key apoptotic molecules affecting the death receptor- or mitochondria-mediated cell death pathways. Indeed, deficiency of ORAI1 dramatically decreased the mRNA expression levels of FasL, not Fas, as judged by measurements of FasL transcripts, which is consistent with the decrease of surface-expressed FasL proteins in Orai\(^{1/-}\) T cells (Fig. 4A). However, lower expression of FasL in Orai\(^{1/-}\) T cells may not be the sole contributor for their high resistance to cell death because T cells from Fasl\(^{-/-}\) mice lacking expression of Fas, therefore harboring disabled Fas/FasL signaling, did not show the same growth pattern upon repetitive stimulation as observed in Orai\(^{1/-}\) T cells (Supplemental Fig. 3). These results suggest that the impact of ORAI1 deficiency can be broader than just a decrease in expression of Fas ligand. Hence, we examined expression levels of various molecules known to be involved in T cell death. Our analysis showed that the mRNA expression levels of a proapoptotic factor Noxa was severely impaired in Orai\(^{1/-}\) T cells, whereas that of Bim was mildly influenced (Fig. 4B). The transcript levels of other proapoptotic molecules such as Bok and Bak also showed reduced expression in Orai\(^{1/-}\) T cells, whereas the mRNA expression levels of the antiapoptotic molecule, Bcl-2, was not affected. Correspondingly, the protein levels of these proapoptotic molecules including Bok and Bax were also reduced in Orai\(^{1/-}\) effector T cells while Bim was marginally increased in the absence of ORAI1 (Fig. 4C). The protein levels of Noxa could not be tested due to lack of the Ab that can detect the murine Noxa protein (56). Among antiapoptotic candidates, Mcl-1 protein levels were increased in ORAI1-deficient T cells, whereas Bcl-2 levels remained unaffected, which is consistent with the mRNA analyses (Fig. 4C). Recently, it was proposed that the ratio of Noxa to Mcl-1 is important for setting up a threshold for cell death induced by TCR stimulation (56). T cells lacking Noxa (Pmaip\(^{1/-}\)) show a survival advantage.
ORAI1 deficiency reduces nuclear translocation of NFAT upon stimulation and resistance of ORAI1-deficient T cells to cell death is recovered by expression of CA-NFAT

So far, our data suggest that Ca\textsuperscript{2+} entry via ORAI1 induces T cell death by influencing the transcription levels of multiple apoptotic genes, and this event can be mediated by transcription factors that are activated by elevated [Ca\textsuperscript{2+}]\textsubscript{i} (e.g., NFAT). To examine a role of NFAT in ORAI1-mediated cell death, first we measured the nuclear accumulation of NFAT in ORAI1-deficient T cells. In WT cells, most of the NFAT proteins translocated into the nuclei after treatment with 1 μM of ionomycin (Fig. 5A, top panels). Even at the lowest concentration of ionomycin that we tested (0.2 μM), translocation was observed, albeit with less efficiency. However, treatment of ORAI1-deficient T cells with even high concentrations of ionomycin (1 μM) resulted in reduced levels of nuclear NFAT (Fig. 5A, bottom panels). As seen in Fig. 5B, within 30 min of ionomycin (1 μM) treatment, only ~50% of ORAI1-deficient cells showed nuclear NFAT, whereas >80% of WT cells showed nuclear NFAT. Even after 2 h, a majority of the WT cells (65%) retained nuclear NFAT, but only a small fraction of ORAI1-deficient cells (~25%) showed nuclear NFAT (Fig. 5B). These results were validated by immunoblotting to detect dephosphorylated NFAT in *Orai*\textsuperscript{+/+} and *Orai*\textsuperscript{−/−} cells after stimulation with anti-CD3 Ab and ionomycin (Fig. 5C). We observed a robust dephosphorylation of NFAT after stimulation with anti-CD3 Ab (20 μg/ml) and ionomycin (1 μM) in WT cells, which was dramatically reduced in ORAI1-deficient cells. Consistent with reduced NFAT translocation, ORAI1-deficient T cells showed a substantial reduction in production of IL-2 and IFN-γ after stimulation with anti-CD3 and anti-CD28 Abs (Supplemental Fig. 4). Together, these results suggest a close correlation between ORAI1-mediated Ca\textsuperscript{2+} entry and activation of NFAT, and the resistance of *Orai*\textsuperscript{−/−} T cells to cell death can be partially attributed to reduced translocation of NFAT.

Next, to examine if NFAT activity was directly responsible for resistance to cell death observed in ORAI1-deficient cells, we measured cell death upon expression of WT and the CA mutant of NFAT. CA-NFAT with alanine substitutions of 12 key serine/threonine residues exists in a dephosphorylated form and resides in the nucleus (23). WT T cells showed robust cell death upon stimulation with anti-CD3 Ab and ionomycin (Fig. 5D, left panel). Over-expression of WT-NFAT marginally enhanced the levels of cell death, whereas expression of the CA-NFAT induced cell death even under resting conditions, which was pronounced after stimulation. In ORAI1-deficient cells, whereas expression of WT-NFAT did not enhance cell death, CA-NFAT induced cell death under both resting as well as stimulated conditions (Fig. 5D, right panel). These results strongly suggest that NFAT plays a major role in the cell death pathways induced by Ca\textsuperscript{2+} entry via ORAI1. A proportional relationship between the intracellular Ca\textsuperscript{2+} levels and cell death in T cells

Next, we sought to determine the correlation between intracellular Ca\textsuperscript{2+} and induction of cell death by expressing various mutants of *Orai*1 in WT and ORAI1-deficient T cells. The goals of these experiments were 2-fold. First, we wanted to verify whether expression of ORAI1 in ORAI1-deficient T cells can rescue their reduced cell death phenotype. Second, we wanted to examine whether a further reduction in SOCE in ORAI1-deficient T cells can enhance their resistance to cell death. We chose *Orai1*\textsuperscript{E106Q} and *Orai1*\textsuperscript{R91W} as ORAI1 mutants that result in inactive CRAC channels (5). Expression of *Orai1*\textsuperscript{E106Q} and *Orai1*\textsuperscript{R91W} strongly inhibited SOCE in WT

FIGURE 4. ORAI1 deficiency suppresses death receptor- and mitochondria-mediated cell death pathways at a transcriptional level. A. mRNA expression analysis of effector CD4\textsuperscript{+} T cells from 6-wk-old *Orai*\textsuperscript{+/−} and *Orai*\textsuperscript{−/−} mice that were left unstimulated (Resting) or stimulated (Stim) with plate-bound 1 μg/ml anti-CD3 for 6 h (top panels). The mRNA expression levels of FasL and Fas were analyzed by quantitative RT-PCR. The data represent average ± SD from three independent measurements performed in triplicates. Asterisks (*) indicate statistically significant differences in expression levels between *Orai*\textsuperscript{+/−} and *Orai*\textsuperscript{−/−} T cells. Surface expression of Fas-L in *Orai*\textsuperscript{+/+} and *Orai*\textsuperscript{−/−} T cells (bottom panels). Effector T cells cultured without skewing Abs were left unstimulated (Res; gray) or restimulated with 2 μg/ml plate-coated anti-CD3 Ab for 16 h (Stim; black histograms), stained for Fas-L, and analyzed by flow cytometry. B. The expression levels of key pro- and antiapoptotic molecules involved in T cell death were analyzed by quantitative RT-PCR. The data represent average ± SD from three independent measurements performed in triplicate. Asterisks indicate transcripts showing a significant difference in expression levels between *Orai*\textsuperscript{+/−} and *Orai*\textsuperscript{−/−} T cells. C. Whole-cell lysates from naïve (N), resting (E), and stimulated effector T cells (S, anti-CD3 Ab, 1 μg/ml for 6 h) were immunoblotted for various pro- and antiapoptotic proteins. The numbers below the bands represent normalized band intensities after comparison with β-actin levels. A representative of four independent experiments is shown. Asterisks indicate proteins showing a significant difference in expression levels between *Orai*\textsuperscript{+/+} and *Orai*\textsuperscript{−/−} T cells.

in vitro, resulting in an increase in the number of effector and memory T cells in vivo (56). Our results show a novel role of ORAI1-mediated Ca\textsuperscript{2+} entry in regulating the ratio of Noxa to Mcl-1 (Fig. 4B, 4C). In summary, our data suggest that reduced [Ca\textsuperscript{2+}]\textsubscript{i} in *Orai*\textsuperscript{−/−} T cells after stimulation decreased cell death and enhanced cell survival by influencing multiple cell death pathways including FasL expression and the ratio of Noxa to Mcl-1.
T cells (Fig. 6A, left panel). These data showed that ORAI1\textsuperscript{R91W} in addition to ORAI1\textsuperscript{E106Q} also has a strong suppression effect on the endogenous CRAC channel activity when expressed in WT T cells. In ORAI1-deficient T cells, expression of WT ORAI1 recovered SOCE, whereas that of ORAI1\textsuperscript{E106Q} and ORAI1\textsuperscript{R91W} further suppressed the residual SOCE in these cells, most likely by multimerizing with other ORAI proteins (Fig. 4A, right panel).

Next, we examined the effect of expression of these mutants on cell death. As expected, in WT T cells, expression of ORAI1\textsuperscript{R91W} reduced cell death upon anti-CD3 stimulation (Fig. 6B, top two panels). Interestingly, in ORAI1-deficient T cells, expression of WT ORAI1 recovered cell death to the levels similar to WT T cells, whereas expression of ORAI1\textsuperscript{R91W} further suppressed cell death (Fig. 6B, bottom two panels). These results indicate a direct correlation between intracellular Ca\textsuperscript{2+} accumulation via ORAI1 and resistance to cell death, with reduction in SOCE proportionally enhancing resistance to cell death.

ORAI1-deficient T cells showed resistance to cell death and enhanced survival in vivo

T cells from \textit{Oral1}\textsuperscript{−/−} mice showed a strong resistance to cell death upon stimulation in vitro. To examine if this was the case in vivo, we injected anti-CD3 Ab into \textit{Oral1}\textsuperscript{+/+} and \textit{Oral1}\textsuperscript{−/−} mice and examined depletion of T cells. In the lymph nodes of \textit{Oral1}\textsuperscript{+/+} mice, CD4\textsuperscript{+} population decreased upon injection of anti-CD3 Ab; however, the depletion was much less in \textit{Oral1}\textsuperscript{−/−} mice (Fig. 7A, 7B). Depletion of CD8\textsuperscript{+} cells in control mice was more pronounced than CD4\textsuperscript{+} cells and even CD8\textsuperscript{+} cells survived better in \textit{Oral1}\textsuperscript{−/−} mice after anti-CD3 Ab injection (Fig. 7B). The number of thymocytes in anti-CD3 Ab-injected \textit{Oral1}\textsuperscript{+/+} control mice reduced markedly (25% of that in PBS-injected mice), whereas that in \textit{Oral1}\textsuperscript{−/−} mice decreased less (50% of that in PBS-injected mice) (Fig. 7C), recapitulating in vitro resistance to cell death (Fig. 2A, 2B). These results further support the idea that ORAI1-mediated cell death upon stimulation can be a common mechanism for CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the peripheral lymphoid organs as well as thymocytes.

To further test the hypothesis that reduced cell death of \textit{Oral1}\textsuperscript{−/−} T cells may benefit T cell survival in vivo, we transferred \textit{Oral1}\textsuperscript{+/+} and \textit{Oral1}\textsuperscript{−/−} T cells into immunocompromised NSG mice. After transfer of the same number of CFSE-labeled cells (3 \times 10\textsuperscript{6}) into NSG recipients i.v. injection, we detected only 0.3% of the total splenocytes of the recipient mice as \textit{Oral1}\textsuperscript{+/+} CFSE-positive cells after 1 wk, whereas 3-fold more \textit{Oral1}\textsuperscript{−/−} CFSE-positive cells were detected (9.9% of total splenocytes; Fig. 7D). These results were confirmed independently by noninvasive imaging of transferred \textit{Oral1}\textsuperscript{+/+} and \textit{Oral1}\textsuperscript{−/−} CD4\textsuperscript{+} T cells expressing firefly luciferase. Under identical conditions of isolation, transduction, and transfer, \textit{Oral1}\textsuperscript{−/−} T cells showed enhanced survival over a period of 3 wk when compared with \textit{Oral1}\textsuperscript{+/+} T cells (Fig. 7E). Taken together, these results suggest that deficiency of ORAI1 antagonizes cell death and improves survival of T cells in vitro and in vivo.

**Discussion**

Ca\textsuperscript{2+} ions play a pivotal role in cell proliferation and death by controlling a plethora of signaling pathways (40). Although in vitro pharmacological studies suggest a direct role of Ca\textsuperscript{2+} in cell death after TCR stimulation, the exact route of Ca\textsuperscript{2+} entry and the...
These results suggest that the increase in SP population in aged (TG) in the presence of extracellular solution containing 2 mM Ca\textsuperscript{2+}. Each channel activity was determined after store depletion with thapsigargin with retroviruses encoding cDNAs for WT or ORAI1 mutants. CRAC expression of ORAI1R91W.

In this study, we reaffirmed the important role of [Ca\textsuperscript{2+}] for T cell a lack of the molecular identity and an appropriate animal model. Detailed molecular mechanism have not been investigated due to a lack of the molecular identity and an appropriate animal model. In this study, we reaffirmed the important role of [Ca\textsuperscript{2+}], for T cell death using Orai1\textsuperscript{−/−} T cells and expression of the dominant-negative mutants of ORAI1 in WT T cells. In addition, we demonstrated that the ORAI1–NFAT pathway is crucial for activation and to maintain the size of the T cell pool by playing a negative role in T cell survival.

RESULTS

Recovery of activated T cell death in ORAI1\textsuperscript{−/−} T cells by expression of WT ORAI1 and suppression of [Ca\textsuperscript{2+}] entry and cell death by expression of ORAI1\textsuperscript{R91W}. A, WT or Orai1\textsuperscript{−/−} T cells were transduced with retroviruses encoding cDNAs for WT or ORAI1 mutants. CRAC channel activity was determined after store depletion with thapsigargin (TG) in the presence of extracellular solution containing 2 mM Ca\textsuperscript{2+}. Each trace represents average ± SEM from 50–60 individual cells. B, Naive T cells were stimulated with plate-bound anti-CD3 and anti-CD28 Abs and transduced with the viruses encoding WT ORAI1 (WT-ORAI1) or the R91W mutant (ORAI1\textsuperscript{R91W}). After 4 d, T cells were restimulated with plate-coated anti-CD3 Ab (1 μg/ml) for 6 h, and cell death was assessed by Annexin V and 7-AAD staining. Representative data from three independent experiments are depicted. In these experiments, only GFP\textsuperscript{+} T cells were selected for analysis.

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that the [Ca\textsuperscript{2+}] levels determine cell death or maturation of thymocytes, depending on avidity of Ag–TCR interactions and the strength of costimulation (e.g., CD28) (42, 46–48). Together, these results suggest that high [Ca\textsuperscript{2+}], through ORAI1 may be commonly used to control the negative selection process in the thymus and to maintain the size of the T cell pool by playing a negative role in T cell survival.
Our and other groups’ observations showed that CRAC channels are crucial for the function of effector T cells such as cytokine production (Supplemental Fig. 4) (13, 14). These data together with the SCID symptoms of human patients harboring mutations in ORAI1 or STIM1 genes demonstrate a positive role of CRAC channels in the immune response (5, 12, 57). In this study, we showed that ORAI1 plays a bona fide role in stimulation-induced cell death, further emphasizing the role of ORAI1 in the diverse functions of effector T cells in addition to cytokine production (Fig. 1). So far, none of the data from the patients and mice harboring deletion or mutations of Orai1 and STIM1 genes indicates any severe defect in development or homing of T cells in the peripheral lymphoid organs. However, these results do not rule out the role of Ca\(^{2+}\) signaling in T cell development or homing because it is still possible that Ca\(^{2+}\) plays a role via entering through alternate routes (e.g., ORAI2, ORAI3, or other non–store-operated Ca\(^{2+}\) channels) instead of ORAI1. In support of this idea, reduction of SOCE in ORAI1-deficient naive T cells was much less than that in effector T cells, and ORAI2 instead of ORAI1 was abundantly expressed in naive T cells as shown by mRNA expression analysis (13, 14).

The role of CRAC channels in T cell proliferation is more complex than in cytokine production or cell death because it differs significantly in various mouse models. In the current work, we showed that Orai1–/– T cells do not have a defect in proliferation (Fig. 1). Instead, the absence of ORAI1 enhanced the proliferation rate at the early stage of activation. A recent report examining T cell functions of knockin mice harboring a point mutation at position 93 (ORAI1R93W) to recapitulate the phenotype observed in SCID patients (ORAI1R91W) showed markedly reduced proliferation of Orai1R93W/Orai1R91W effector T cells (58). It is known that ORAI1 can form heteromultimers with other Orai family members (6), and human ORAI1R91W mutant has been shown to function in a dominant negative manner in an ORAI1-STIM1 overexpression system (59). We also observed suppression of SOCE in WT and Orai1–/– T cells upon expression of ORAI1R91W (Fig. 6A). Therefore, it is possible that the levels of SOCE in T cells isolated from Orai1R93W/Orai1R91W mice are much lower than those of Orai1–/– T cells may not reach the minimum requirement of [Ca\(^{2+}\)], to promote proliferation of T cells. Consistent with these observations, STIM1-deficient T cells showed normal proliferation with marginal differences in vitro and in vivo after immunization, whereas T cells lacking both STIM1 and STIM2 proliferate to a much lesser extent (18, 19). Together, these results suggest that T cell proliferation does not require high [Ca\(^{2+}\)] levels, and the threshold of [Ca\(^{2+}\)] necessary for T cell proliferation seems to be much lower than that for cytokine production or cell death.

A direct correlation between the [Ca\(^{2+}\)] levels and activated T cell death was further verified by recovery experiments in ORAI1-deficient T cells. Expression of ORAI1 in ORAI1-deficient T cells almost completely recovered normal cell death levels in addition to SOCE (Fig. 6). Interestingly, we were able to suppress T cell death in WT T cells by expression of the dominant-negative mutant of Orai1, ORAI1E106Q, and ORAI1E106Q (Fig. 6B and data not shown). Expression of ORAI1E106Q in ORAI1-deficient T cells further suppressed the residual levels of SOCE and cell death induced by stimulation (Fig. 6B). Together, these results indicate that the degree of stimulation-induced T cell death is directly proportional to [Ca\(^{2+}\)] levels regulated via ORAI1. Furthermore, our data support a model in which excessive Ca\(^{2+}\) entry mediated by ORAI1 supports death programs mediated by multiple cell death pathways, including a transcriptional event mediated by NFAT and nontranscriptional events of mitochondrial Ca\(^{2+}\) uptake. This conclusion is supported by the following observations. First, Orai1–/– T cells showed reduced expression of proapoptotic molecule FasL that is known to be regulated by Ca\(^{2+}\)-NFAT signaling. Second, our results showed [Ca\(^{2+}\)]\(_{i}\)-mediated regulation of the ratio of Noxa to Mcl-1, which is recently identified to set a threshold for T cell death (Fig. 4) (56). Finally, Orai1–/– T cells showed severely reduced mitochondrial Ca\(^{2+}\) uptake and thereby reduced mitochondria-mediated cell death (Fig. 3). Because the NFAT family of transcription factors are direct downstream targets of Ca\(^{2+}\)-CaM/calcineurin pathway, our results together with those of NFAT family-deficient mice suggest that excessive [Ca\(^{2+}\)]\(_{i}\) turns on cell death transcriptional programs, many of which are regulated by NFAT (23, 55). It is also known that predominant accumulation of nuclear NFAT can turn on transcriptional programs of a status of T cell tolerance termed anergy (23). ORAI1-deficient T cells were not anergic because proliferation and cell cycle progression were actively induced upon repetitive stimulations (Fig. 1A). Therefore, the threshold levels of [Ca\(^{2+}\)]\(_{i}\) for anergy induction may be higher than those observed in Orai1–/– T cells. Future studies in Orai1–/– T cells examining the expression levels or activities of anergy-inducing factors including E3 ubiquitin ligases (Cbl-b, Itch, or ORA1) and caspase 3 that cleaves/degrades TCR signaling molecules and the zinc finger transcription factor Ikaros that suppresses IL-2 transcription would validate the role of ORAI1 in anergy (60, 61).

The rise in [Ca\(^{2+}\)]\(_{i}\) triggered by TCR stimulation plays a pivotal, positive role in T cell activation, cytokine production, and proliferation. However, symptoms of the patients with nonfunctional CRAC channels, especially those with mutations in STIM1 gene, are perplexing because in addition to a defect in activation of immune cells as exemplified by SCID symptoms, they also display lymphoproliferative disorders demonstrated by lymphadenopathy, splenomegaly, and elevated memory T cell population (12). In addition to the proposed mechanism of reduced regulatory T cell population (18), our observations of resistance to cell death can serve as an alternate explanation for the lymphoproliferative symptoms of SCID patients. Our findings demonstrate a crucial role of high [Ca\(^{2+}\)]\(_{i}\), in diverse immune tolerance mechanisms such as negative selection, induction of cell death, and T cell survival. Our and other groups’ results point toward different threshold levels of [Ca\(^{2+}\)]\(_{i}\), to trigger various functions of T cells including proliferation, anergy, or death. A comprehensive study of animal models with various levels of CRAC channel activity can facilitate further understanding of how different amplitudes of Ca\(^{2+}\) entry influence diverse activities of T cells.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

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Supplementary Figures

Supplementary Figure 1 Cell cycle analysis of ORAI1-deficient T cells after stimulation

(A) Cell cycle progression was analyzed using propidium iodide staining of Orai1^+/+ and Orai1^-/- T cells at 24 and 48 hours post stimulation with anti-CD3 and anti-CD28 antibodies. A representative of three independent experiments is shown. P values were determined using paired Student’s t-test.

(B) Live cell gating using forward/side scattering of WT and ORAI1-deficient (KO) effector T cells after stimulation with plate-bound anti-CD3 and anti-CD28 antibodies. Isolated naive T cells were stimulated, cultured for five days, and re-stimulated. These cells were analyzed for CSFE-labeling experiments in Fig. 1B.

Supplementary Figure 2 Store-operated Ca^{2+} entry in CD4^+ T cells from Orai1^+/+, Orai1^+/−, and Orai1^-/- mice

(A) CRAC channel activity in effector CD4^+ T cells was determined after store depletion with thapsigargin (TG) in the presence of extracellular solution containing 0.5 and 2 mM Ca^{2+}. The bar graph on the right shows average ± s.e.m. from three independent experiments.

(B) CRAC channel activity in effector CD4^+ T cells measured after store depletion by cross-linking of anti-CD3 antibody. Cells were pretreated with biotin-conjugated anti-CD3 antibody and streptavidin was added at the indicated time points to induce cross-linking. The bar graph on the right shows average ± s.e.m. from three independent experiments.

Supplementary Figure 3 Proliferation of T cells from Faslpr mice upon repetitive stimulations

Naïve CD4^+ T cells purified from control (MRL) and MRL-Faslpr mice were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml) antibodies for 2 days. At day 6 post stimulation, T cells were restimulated with plate-bound anti-CD3 (0.5 μg/ml) and soluble anti-CD28 (1 μg/ml) antibodies for 24 hours and counted for live cells. The same condition was used for successive stimulation of Faslpr T cells.

Supplementary Figure 4 cytokine production of ORAI1-deficient effector CD4^+ T cells

Naïve CD4^+ T cells purified from Orai1^+/+ and Orai1^-/- mice were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml) antibodies for 2 days. At day 6 post stimulation, T cells were restimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml) antibodies for 6 hours, fixed, and intracellularly stained for IL-2 and IFN-γ.
A

WT

KO

S-G2/M

24 hrs

6.32%

11.1%

24 hrs

48 hrs

48.3%

59.5%

24 hrs

% Cells (S-G2/M)

Day1

Day 2

% Cells (G1)

Day 1

Day 2

Effector T cells: Stimulation with anti-CD3/CD28 Ab

B

Suppl Fig. 1
Suppl Fig. 3
Suppl Fig. 4