

ORAI1-mediated calcium influx is required for human cytotoxic lymphocyte degranulation and target cell lysis

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Lymphocytes mediate cytotoxicity by polarized release of the contents of cytotoxic granules toward their target cells. Here, we have studied the role of the calcium release-activated calcium channel ORAI1 in human lymphocyte cytotoxicity. Natural killer (NK) cells obtained from an ORAI1-deficient patient displayed defective store-operated Ca^{2+} entry (SOCE) and severely defective cytotoxic granule exocytosis leading to impaired target cell lysis. Similar findings were obtained using NK cells from a stromal interaction molecule 1-deficient patient. The defect occurred at a late stage of the signaling process, because activation of leukocyte functional antigen (LFA)-1 and cytotoxic granule polarization were not impaired. Moreover, pharmacological inhibition of SOCE interfered with degranulation and target cell lysis by freshly isolated NK cells and CD8^+ effector T cells from healthy donors. In addition to effects on lymphocyte cytotoxicity, synthesis of the chemokine macrophage inflammatory protein-1 β and the cytokines TNF- α and IFN- γ on target cell recognition was impaired in ORAI1-deficient NK cells, as previously described for T cells. By contrast, NK cell cytokine production induced by combinations of IL-12, IL-15, and IL-18 was not impaired by ORAI1 deficiency. Taken together, these results identify a critical role for ORAI1-mediated Ca^{2+} influx in granule exocytosis for lymphocyte cytotoxicity as well as for cytokine production induced by target cell recognition.

primary immunodeficiency | cytotoxic lymphocytes | lytic granules | perforin

Cytotoxic lymphocytes, such as CD8^+ T cells and natural killer (NK) cells, can kill virus-infected or transformed cells through polarized release of the contents of cytotoxic granules (1, 2). On recognition of target cells via the T-cell receptor or activating NK cell receptors, cytotoxic granules are anchored to microtubules and migrate toward the immune synapse, together with the microtubule organizing center (MTOC) (3, 4). Polarized cytotoxic granules fuse with the cell membrane, leading to release of the granule content into the synaptic cleft. The pore-forming protein perforin then provides granzymes access to target cells, where they induce apoptotic cell death (5).

Uptake of extracellular Ca^{2+} is required for lymphocyte cytotoxicity (6–8). Vesicle exocytosis in a cytotoxic T-cell [cytotoxic T-lymphocyte (CTL)] line has been described to require a Ca^{2+} current with calcium release-activated calcium (CRAC) channel characteristics (9). The nature of the Ca^{2+} channel that mediates Ca^{2+} influx and cytolytic function in cytotoxic lymphocytes has not been determined (2, 10). Moreover, the involvement of Ca^{2+} influx in signaling processes mediating target cell adhesion, cytolytic granule polarization, and exocytosis is not clear. In a remarkable series of experiments, ORAI1 and stromal interaction

molecule 1 (STIM1) were identified as the molecular constituents of the CRAC channel in T cells (11–14). STIM1, localized in the endoplasmic reticulum (ER), acts as the sensor of Ca^{2+} depletion from the ER, and its physical interaction with ORAI1 triggers the opening of CRAC channels and influx of extracellular Ca^{2+} , a process termed store-operated Ca^{2+} entry (SOCE) (15). The key role of this pathway in immune cell function is illustrated by the severe immunodeficiency in patients with impaired CRAC channel function attributable to mutations in *ORAI1* and *STIM1* (13, 16, 17). Ca^{2+} influx is required for the activation of the transcription factor nuclear factor of activated T cells (NFAT), which, in turn, is essential for the expression of cytokines, such as IL-2. This pathway is defective in T cells from ORAI1-deficient patients (18).

Here, we have used NK cells from two patients who are ORAI1-deficient or STIM1-deficient, respectively, as well as CTLs and NK cells from healthy donors treated with inhibitors of SOCE to study the role of ORAI1 in lymphocyte cytotoxicity. Our results demonstrate that SOCE mediated via ORAI1 is critical for target cell-induced lytic granule exocytosis in NK cells and CTLs as well as for target cell-induced proinflammatory chemokine and cytokine production by human NK cells.

Results

ORAI1-Deficient NK Cells Show Defective SOCE. During reevaluation of a 15-y-old ORAI1-deficient patient (19) who had received a hematopoietic stem cell transplant at the age of 4 mo, cell-specific chimerism was determined. A short tandem repeat (STR) analysis was performed at 15 loci using DNA from whole blood from donor and recipient before transplantation and from sorted NK-, B-, and T-cell populations posttransplantation (Fig. 1A). After transplantation, the pattern of STRs in NK cells showed exclusively recipient signals, whereas there was a small donor signal among B and T cells (Fig. 1B). This indicates a mixed chimerism in the patient with a maximum of about

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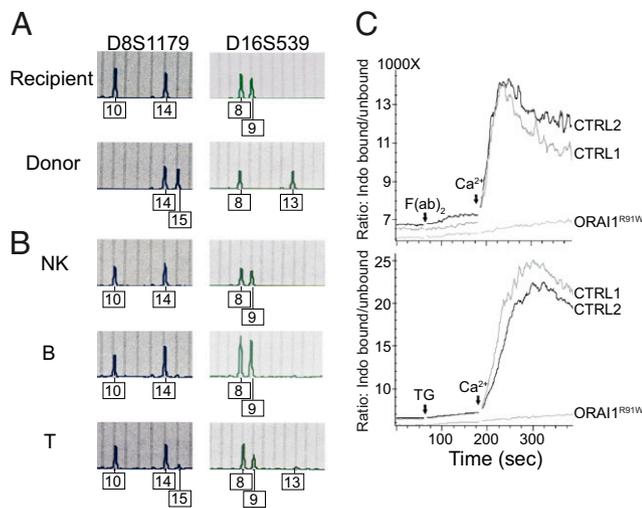


Fig. 1. ORAI1-deficient NK cells have impaired SOCE influx. (A) Split chimerism of lymphocyte subpopulations. STR analysis was performed at the indicated markers using DNA isolated from PBMCs of the patient (recipient) and the bone marrow donor before transplantation (A) and DNA isolated from the indicated purified cell populations after transplantation (B). Although there is a small donor signal (15 in locus 1 and 13 in locus 2) among T cells and a minimal donor signal among B cells, all NK cells are of recipient origin. (C) Ca²⁺ influx in stimulated NK cells. Purified NK cells from the patient (ORAI1^{R91W}) and two healthy donors (CTRL) were either preincubated with anti-CD16 mAb in Ca²⁺-free PBS, followed by cross-linking and the addition of 2 mM CaCl₂ (Upper), or incubated with 1 μM TG, followed by the addition of 2 mM CaCl₂ (Lower). The graph shows the ratio of unbound to bound indo-1-AM as a measure of Ca²⁺ influx during the course of the experiment. The assay was performed twice with similar results. CTRL, control; TG, thapsigargin.

10–15% donor T cells and 5% donor B cells, whereas all his NK cells were of host origin carrying the R91W single amino acid substitution in ORAI1 (Fig. 1B). To analyze the consequences of this mutation for SOCE in NK cells, changes in intracellular Ca²⁺ levels were measured in purified NK cells following the passive depletion of intracellular Ca²⁺ stores. For this purpose, we either treated NK cells with thapsigargin (Fig. 1C, Lower), which depletes ER Ca²⁺ stores through inhibition of the sarco/endoplasmic Ca²⁺ ATPase, or activated NK cells through cross-linking of CD16 (Fig. 1C, Upper), which causes emptying of ER Ca²⁺ stores via phospholipase C-γ activation and phosphoinositide generation. Freshly isolated NK cells were treated with thapsigargin or anti-CD16 mAb in the absence of extracellular Ca²⁺ (Fig. 1C). Readdition of Ca²⁺ to the medium led to robust Ca²⁺ influx in NK cells from healthy donors but not in NK cells from the patient, showing that ORAI1 is essential for SOCE in NK cells.

Notably, the ORAI1-deficient NK cells showed normal frequencies and expression levels of NK-cell activating and inhibitory receptors (Fig. S1), although it should be noted that the frequency of NK cells expressing the activating receptor CD2 and inhibitory receptor KLRG1 was unusually low on the patient's NK cells. ORAI1-deficient NK cells also displayed normal intracellular expression of perforin (Fig. S2).

Lymphocyte Cytotoxicity Is Dependent on ORAI1 and STIM1. Because uptake of extracellular Ca²⁺ is required for the cytolytic activity of CTLs, we next assessed cytotoxicity in ORAI1-deficient NK cells. Freshly purified ORAI1-deficient NK cells failed to lyse Fc receptor (FcR)-positive L1210 target cells after incubation with anti-CD16 mAbs and displayed severely impaired lysis of K562 cells (Fig. 2A), indicating defective antibody-dependent cellular cytotoxicity and natural cytotoxicity. We also studied NK cells from a 5-y-old STIM1-deficient patient carrying a R429C single amino acid substitution resulting in defective SOCE (Fig. S3).

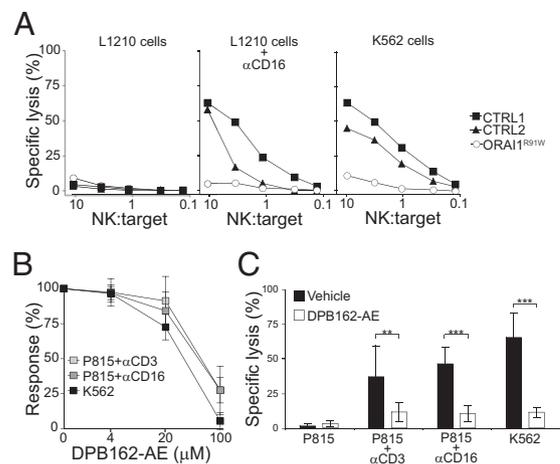


Fig. 2. ORAI1 dependence of lymphocyte cytotoxicity. (A) Cytotoxicity was assessed in a ⁵¹Cr release assay using purified NK cells from the ORAI1-deficient patient (ORAI1^{R91W}) and two healthy donors (CTRL) as effector cells and target cells at the indicated effector/target (NK:target) ratios. For antibody-dependent cellular cytotoxicity, L1210 target cells were either preincubated with anti-CD16 mAb or left unlabeled. For natural cytotoxicity, K562 cells were used as target cells. CTRL, control. (B and C) PBMCs were preincubated with different concentrations of DPB162-AE, as indicated, for 30 min and mixed with ⁵¹Cr-labeled K562 or P815 target cells as indicated. Specific lysis was assessed with an effector/target ratio of 100:1. Results are representative of four independent experiments. (B) Graph depicts the response calculated as the percentage of the specific lysis with different concentrations of DPB162-AE relative to that with vehicle only. Values with error bars represent mean ± SD of five donors. (C) Plots depict specific lysis with or without 100 μM DPB162-AE. Values with error bars represent mean ± SD of nine donors. Results are representative of at least three independent experiments. ***P < 0.001.

STIM1-deficient NK cells also displayed defective lysis of K562 target cells (Fig. S4).

To substantiate these data, we used pharmacological inhibitors of SOCE, DPB162-AE, and 2-APB (20, 21). DPB162-AE is a 2-APB analog recently characterized as a potent inhibitor of SOCE that disrupts puncta formation of STIM1, and thus activation of ORAI Ca²⁺ channels (21). DPB162-AE blocked SOCE in NK cells on readdition of extracellular Ca²⁺ after NK cells had been treated with thapsigargin or had been activated through cross-linking of CD16 (Fig. S5). DPB162-AE significantly inhibited NK cell-mediated lysis of K562 cells and CD16-dependent lysis of P815 cells in a dose-dependent manner (Fig. 2B and C). DPB162-AE also inhibited T cell-mediated anti-CD3-dependent lysis of P815 cells (Fig. 2B and C). Partial inhibition of K562 cell lysis was also observed with 100 μM 2-APB. At these concentrations, the pharmacological inhibitors were not cytotoxic (Fig. S6). Thus, pharmacological inhibition of SOCE abolished lymphocyte cytotoxicity induced by triggering of the T-cell receptor, the FcR CD16, and engagement of ligands for NK cell-mediated natural cytotoxicity.

Inside-Out Signals for LFA-1 Activation and Cytotoxic Granule Polarization Are Not Impaired by ORAI1 Deficiency. NK cell cytotoxicity comprises several steps, including target cell adhesion, granule polarization, and granule exocytosis (22, 23). Target cell adhesion through leukocyte functional antigen (LFA)-1 is augmented by so-called “inside-out signals,” which lead to a conformational change in the ectodomain of LFA-1. Particular mAbs, such as 327C, specifically recognize this extended ligand-binding conformation of LFA-1 (24, 25). We assessed the role of ORAI1 in mediating these inside-out signals by stimulation of ORAI1-deficient CD56^{dim} NK cells with K562 cells or anti-CD16-coated P815 target cells. No defect in inside-out signals for LFA-1 activation was detected (Fig. 3A). Similar results were obtained using a series of previously described *Drosophila* Schneider 2 (S2)-cell

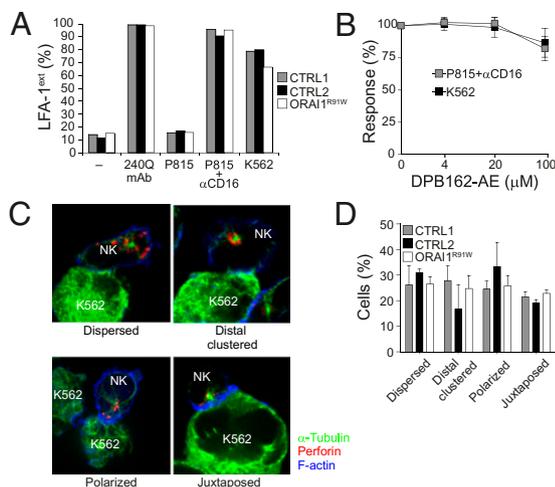


Fig. 3. Signals for LFA-1 activation and granule polarization are ORAI1-independent. (A) PBMCs from the ORAI1-deficient patient (ORAI1^{R91W}) or healthy donors (CTRL) were mixed with target cells as indicated. Cells were incubated for 5 min at 37 °C, stained with lineage marker and conformation-specific biotinylated anti-LFA-1 mAbs, washed, and stained with fluorochrome-conjugated streptavidin. The percentage of CD3⁺CD56^{dim} NK cells with 327C^{high} expression (LFA-1^{ext}), indicating LFA-1 in the extended ligand-binding conformation, is presented. One representative experiment of two is shown. (B) PBMCs from healthy donors were preincubated with different concentrations of DPB162-AE as indicated for 30 min, incubated with target cells, and stained as described in A. The graph depicts the response calculated as the percentage of 327C^{high} CD56^{dim} NK cells with varying concentrations of DPB162-AE relative to that with vehicle only. Values with error bars represent mean \pm SD of four donors. (C and D) Purified NK cells from the ORAI1-deficient patient or healthy donors were mixed and incubated with K562 cells for 20 min, fixed, permeabilized, and stained intracellularly with phalloidin, α -tubulin, and perforin. Thereafter, NK cells in conjugates with target cells were scored for polarization of perforin granules and the MTOC. (C) Representative images of different categories of NK cell–K562 cell conjugates are shown. NK cells were derived from a healthy control. (D) Plot depicts the percentage of NK cells with different degrees of perforin granule and MTOC polarization, as indicated. Values with error bars represent mean \pm SD of more than 190 conjugated NK cells derived from three independent experiments. CTRL, control.

transfectants expressing human intercellular adhesion molecule-1, CD48, ULBP1, or combinations thereof (25) (Fig. S7). Furthermore, in CD56^{dim} NK cells from healthy donors, the ORAI1 inhibitor DBP162-AE did not affect target cell-induced inside-out signals for LFA-1 activation (Fig. 3B).

To address the question of whether granule polarization is dependent on ORAI1, polarization of perforin-containing granules was analyzed in NK cells conjugated to K562 cells. Each NK cell–K562 cell conjugate was scored into one of the following categories: perforin granules and MTOC dispersed distally from target cell synapse (dispersed), perforin granules clustered distally at the MTOC (clustered distally), perforin granules and MTOC polarized toward the target cell synapse (polarized), or perforin granules and MTOC immediately juxtaposed with the MTOC to the target cell synapse (juxtaposed) (Fig. 3C). In the absence of target cells, most NK cells displayed dispersed granules. On conjugation with K562 cells, about 50% of NK cells from healthy donors had perforin granules polarized or immediately juxtaposed to the target cell synapse. A similar pattern of perforin granule polarization was observed in NK cells from the ORAI1-deficient patient (Fig. 3D). The degree of perforin polarization toward K562 cells was similar to what has been reported previously (26). These results indicate that initial signals for adhesion and granule polarization are independent of ORAI1-mediated Ca²⁺ influx in NK cells.

Severely Impaired NK Cell Degranulation in the Absence of ORAI1 or STIM1. Degranulation of cytotoxic lymphocytes can be quantified by determining the surface expression of CD107a following stimulation (27). K562 cells and P815 cells with anti-CD16 mAb induced strong degranulation of control but not of ORAI1-deficient or STIM1-deficient NK cells (Fig. 4A and B and Fig. S8). Similar results were obtained when degranulation was induced through engagement of defined NK cell activation receptors by ligands expressed by S2-cell transfectants (Fig. S9). Furthermore, target cell-induced degranulation of CD56^{dim} NK cells was significantly impaired after pretreatment of cells with the inhibitor DPB162-AE and following coincubation with K562 cells or anti-CD16-coated P815 cells (Fig. 4C and D). A similar degranulation defect was observed in DPB162-AE-pretreated CD8⁺CD62L[−] effector T cells coincubated with P815 cells plus anti-CD3. At a concentration of 100 μ M, 2-APB reduced K562 cell-mediated CD56^{dim} NK cell degranulation by 87%, but did not affect inside-out signals for activation of LFA-1. Taken together, these results demonstrated that cytotoxic lymphocyte degranulation induced by a variety of stimuli required SOCE mediated by STIM1 and ORAI1.

Impaired Cytokine Production of ORAI1-Deficient NK Cells Is Dependent on the Activating Stimulus. Because ORAI1 plays a key role in cytokine production by helper T cells, we also studied the expression of chemokines and cytokines in CD56^{dim} NK cells in response to stimulation by K562 cells or P815 cells plus anti-CD16 mAb. Macrophage inflammatory protein-1 β (MIP-1 β) was expressed in 80–87% of CD56^{dim} NK cells from healthy donors and in 26–41% of CD56^{dim} NK cells from the ORAI1-deficient patient (Fig. 5A and B), indicating a partial dependence of MIP-1 β production on ORAI1. The production of IFN- γ and TNF- α in response to the same stimuli was fully dependent on ORAI1 (Fig. 5A and B). By contrast, stimulation by combinations of IL-12, IL-15, and IL-18 was able to elicit normal production of MIP-1 β and IFN- γ in human ORAI1-deficient CD56^{dim} NK cells (Fig. 5C). These data suggest that target cell recognition by NK cells induces ORAI1-dependent cytokine production, whereas ORAI1 is not required for production of cytokines in response to exogenous cytokine stimuli.

Discussion

This study identifies SOCE mediated by STIM1 and ORAI1 as the key mechanism of extracellular Ca²⁺ influx required for granule exocytosis and cytotoxicity in human cytotoxic lymphocytes. Moreover, a role for ORAI1 in cytokine production by NK cells specifically in response to target cell ligands is documented.

NK cells obtained from an ORAI1-deficient patient and a STIM1-deficient patient had severely impaired natural cytotoxicity. The former patient carries a homozygous mutation in *ORAI1*, leading to a R91W single amino acid substitution. It has previously been shown that this mutation abolishes CRAC channel function in human T cells (13). Our results show that stimulation of NK cells from this patient failed to induce a sustained rise in the concentration of intracellular free calcium. Because of the severe clinical condition of the latter patient, the functional consequences of the *STIM1* mutation could not be fully characterized; however, Ca²⁺ influx in T cells was severely impaired. The defect in Ca²⁺ influx is of similar severity in freshly isolated ORAI1-deficient NK cells as in T-cell lines, suggesting that SOCE in human NK cells is mediated predominantly by ORAI1 and not by other Ca²⁺ channels. Previous studies with T cells from the same patient have established that ORAI1 is required for activation of the transcription factor NFAT; for mitogen-mediated T-cell proliferation; and for the production of cytokines, such as IL-2, IL-4, IFN- γ , and TNF- α , in response to cross-linking of the T-cell receptor or stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (13, 18, 28). Altogether, these results have clearly demonstrated an important role for ORAI1 in T-cell activation. Whether SOCE

focal microscope (Andor) and then scored blinded for the degree of polarization of perforin, MTOC, and F-actin.

Degranulation Assays. NK cell degranulation was assessed as previously described (48). Briefly, 2×10^5 PBMCs were mixed with 2×10^5 target cells in 200 μ L of complete medium. Cells were mixed, spun down for 3 min at $20 \times g$, and incubated for 2 h at 37 °C. Thereafter, the cells were spun down; stained with fluorochrome-conjugated mAbs against CD3, CD56, and CD107a in PBS supplemented with 2% (vol/vol) FBS and 2 mM EDTA for 45 min on ice; washed; resuspended in PBS supplemented with 2% (vol/vol) FBS and 2 mM EDTA; and analyzed by flow cytometry.

Intracellular Cytokine Staining. For stimulation with target cells, 2×10^5 PBMCs were added to 2×10^5 target cells in 200 μ L of complete medium as previously described (46). Briefly, after incubation of the cells for 1 h at 37 °C, Brefeldin A (GolgiPlug; BD Bioscience) was added, followed by an additional 5 h of incubation. In some experiments, cells were stimulated with 10 ng/mL

IL-12 (PeproTech), 100 ng/mL IL-15 (PeproTech), and/or 100 ng/mL IL-18 (R&D Systems). For stimulation with exogenous cytokines, cells were stimulated for 19 h at 37 °C before addition of Brefeldin A and an additional 5 h of incubation. The cells were analyzed on a CyAn ADP LX nine-color flow cytometer (Dako).

Statistical Analysis. Statistical significance was evaluated with a non-parametric Mann–Whitney test using Graphpad Prism software.

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