Cisd2 deficiency impairs neutrophil function by regulating calcium homeostasis via Calnexin and SERCA

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Running Title: Cisd2's Role in Calcium Regulation and Neutrophils
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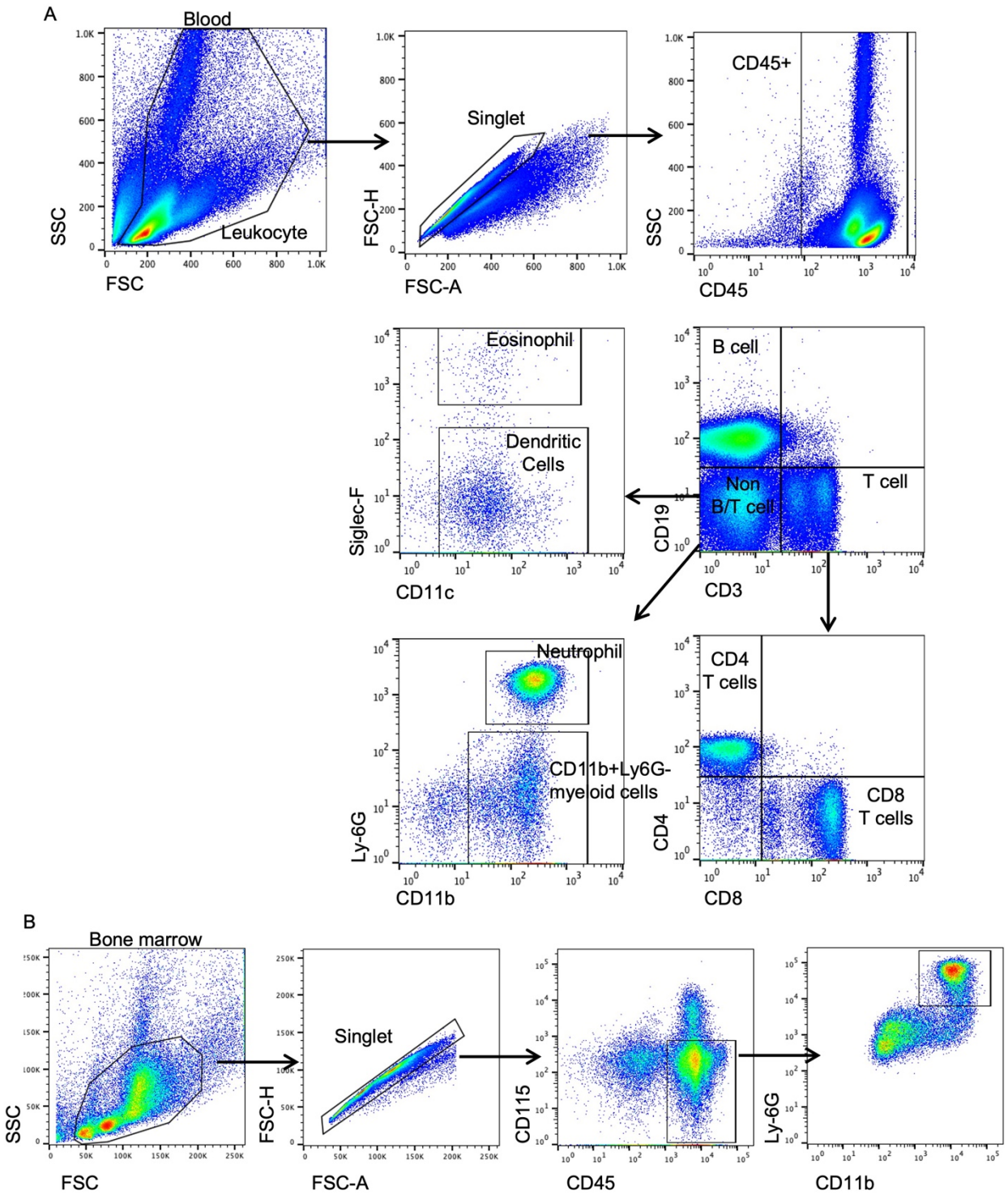
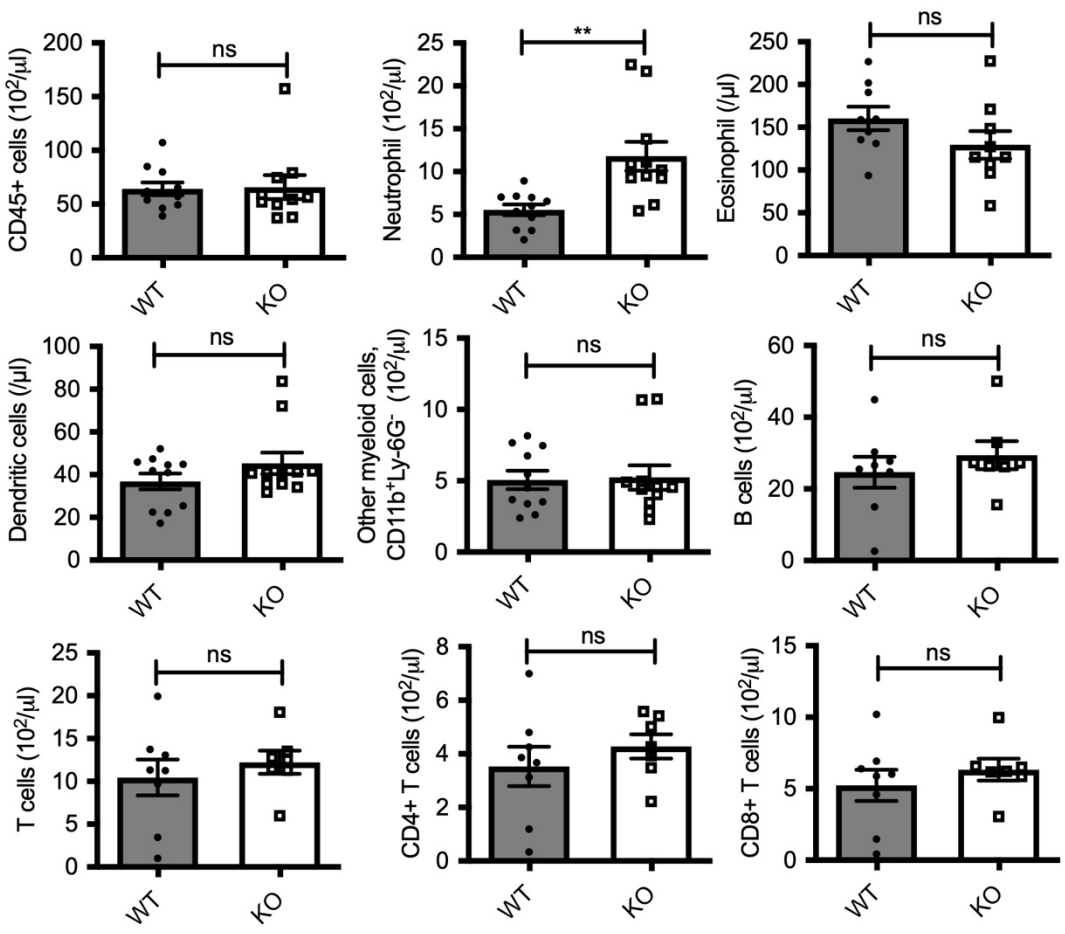
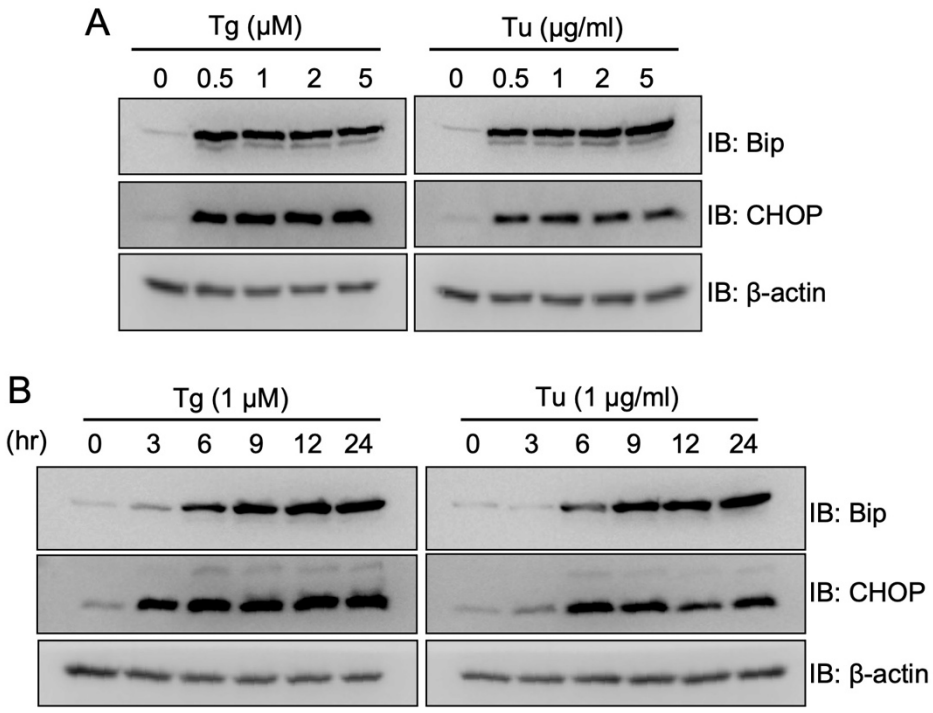


Supplementary Figure 1. (A) Flow cytometry gating strategy for phenotyping general leukocyte subpopulations in blood. An FSC-A/FSC-H gate was used to gate on singlets. Subsequently, a CD45/SSC gate was drawn around leukocytes. Anti-CD19

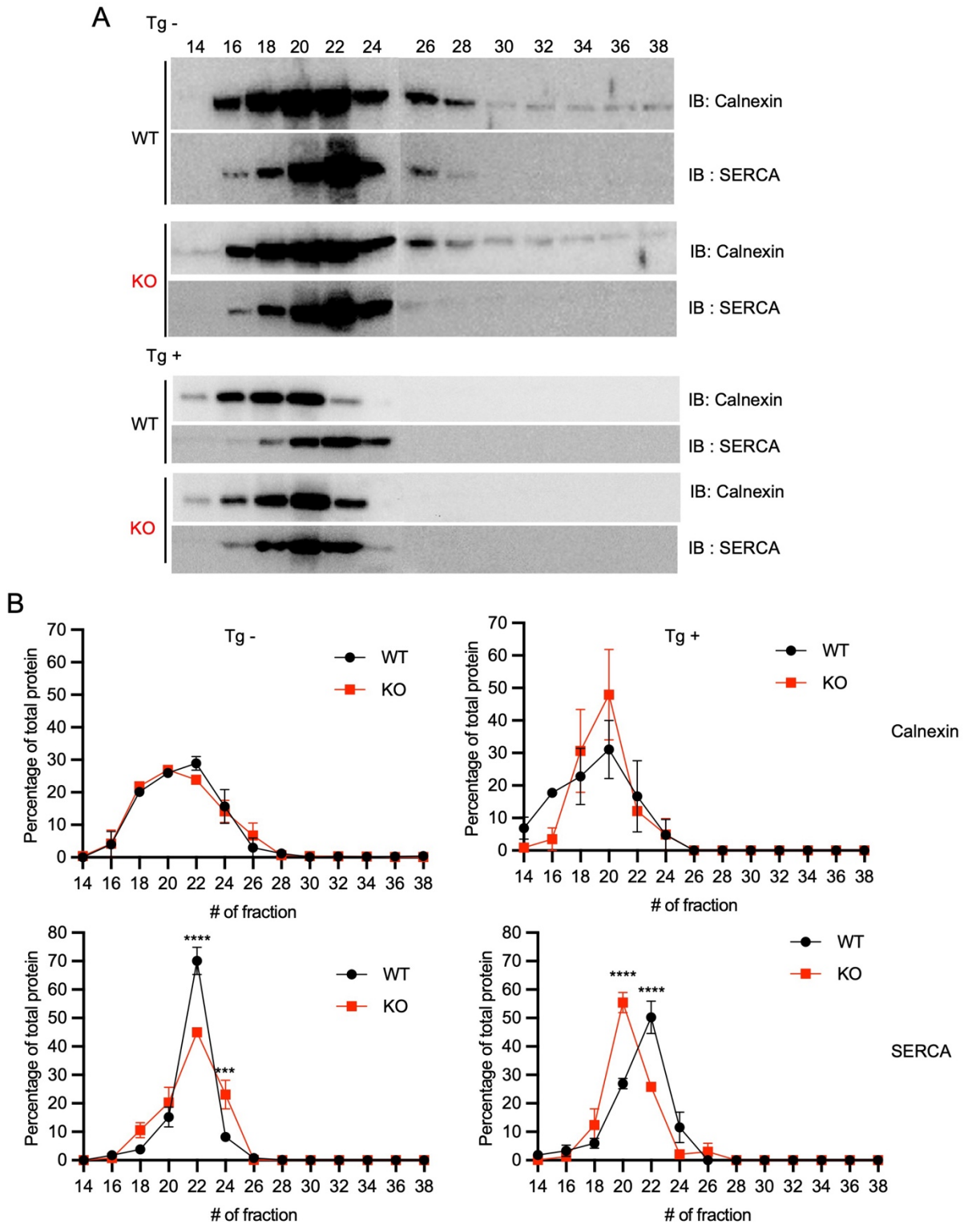
and anti-CD3 were used to identify B, T, and non-B/T cells. Among non-B/T cells, eosinophils, dendritic cells, neutrophils, and CD11b+ Ly6G- myeloid cells were then identified using indicated antibodies. Among T cells, CD4 and CD8 T cells were identified using indicated antibodies. (B) Flow cytometry gating strategy for the identification of neutrophils in bone marrow. An FSC-A/FSC-H gate was used to gate on singlets. Subsequently, CD45+, CD115-, Ly-6G+, CD11b+ cells are identified as neutrophils.



Supplementary Figure 2. Absolute cell counts for blood cells from *Cisd2* knockout and wild-type mice. Symbols refer to individual values for each mouse with the average for each group indicated by bars.

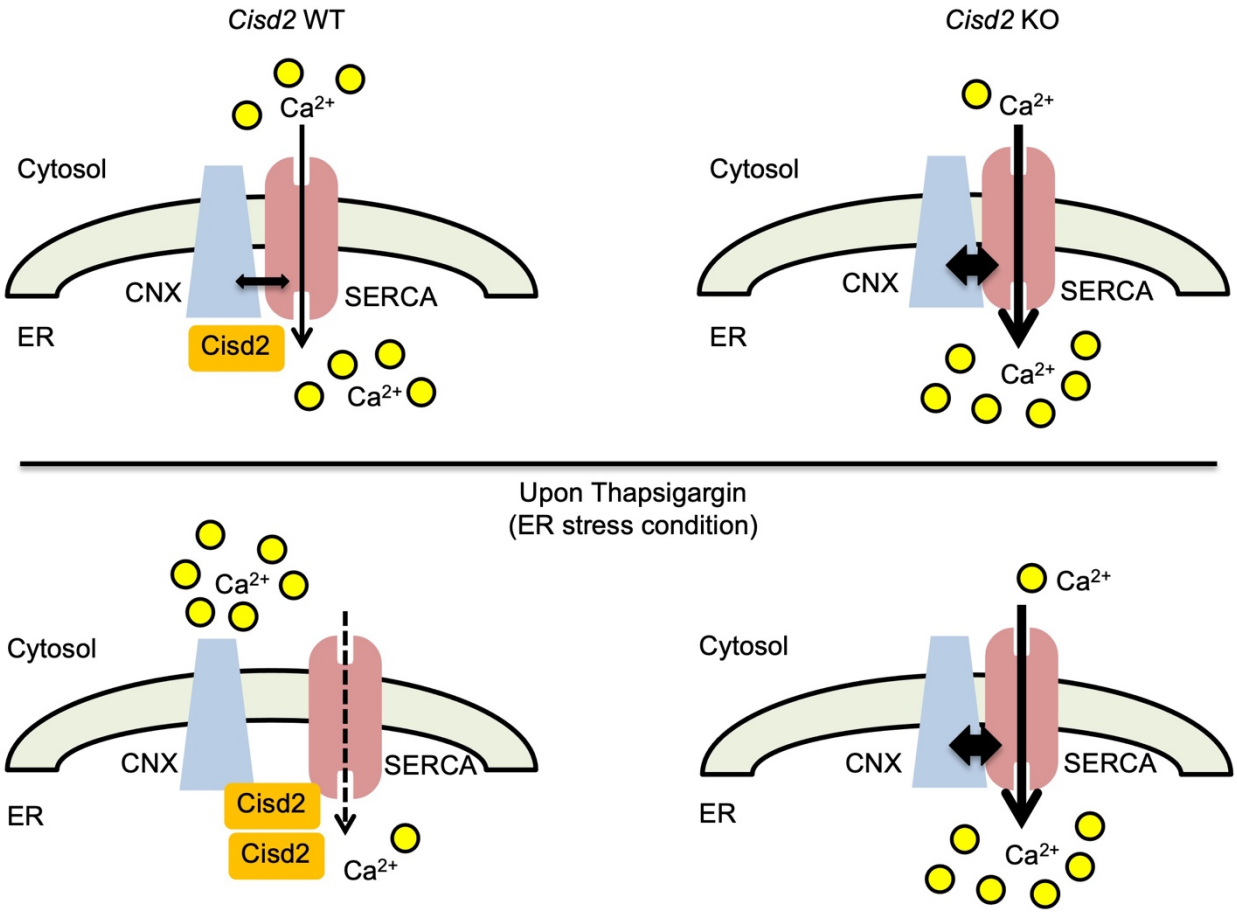


Supplementary Figure 3. ER stress in thapsigarin and tunicamyin treated HEK293T cells. Western blotting of BiP and CHOP in HEK293T cells treated with (A) varying dose of thapsigarin (Tg) and tunicamyin (Tu) and (B) different period of time.



Supplementary Figure 4. Analysis of calnexin and SERCA complex formation in neutrophils under thapsigargin-induced endoplasmic reticulum (ER) stress

conditions. (A) Representative Western blots of SEC fractions (14~38) from neutrophil lysates, prepared with or without Tg (1 μM for 6 h) treatment. Western blotting with anti-Calnexin (CNX), and anti-SERCA. (B) SEC fraction profiles represented as the percentage of Calnexin and SERCA in each fraction, indicating the amount per fraction relative to the total protein amount across all fractions. ***P=0.0002, ****P < 0.0001 by two-way ANOVA.



Supplementary Figure 5. Influence of Cisd2 on Calnexin and SERCA-mediated Ca2+ flux. In wild-type (WT) cells under basal conditions without ER stress, Calnexin binds to SERCA and facilitates calcium transport into the ER. In *Cisd2*-deficient cells, the interaction between Calnexin and SERCA is intensified, potentially leading to a slightly lower cytosolic calcium level. However, upon inhibition of SERCA function by thapsigargin treatment, Calnexin, which aids SERCA activity, dissociates from SERCA and instead forms a stronger bond with Cisd2. This results in a complete inhibition of SERCA function and an elevation in cytosolic calcium levels. Conversely, in *Cisd2*-deficient cells lacking Cisd2 to sequester Calnexin away from SERCA, Calnexin continues to bind to and activate SERCA following thapsigargin treatment.

SUPPLEMENTARY MATERIALS AND METHODS

Cell and animals

Cells were maintained at 37 ° C in a humidified incubator with 5% CO2. HEK293T, Hela and MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Seradigm) and 1% penicillin/streptomycin (Gibco). Cisd2 KO mice were generated and genotyped as previously reported (5). Littermate controls were obtained by crossing Cisd2 heterozygous mice. All experiments were approved and done according to the guidelines of the Institutional Animal Care and Use Committee at the Cleveland Clinic.

Isolation of mouse embryonic fibroblast

Primary MEFs were isolated from *Cisd2* KO and WT littermate control embryos at embryonic day 13.5. The embryos were dissected, and the head and red organs were removed. The remaining tissue was minced and incubated in trypsin-EDTA solution for 30 minutes at 37 ° C. After trypsinization, the cells were suspended in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The MEFs were then cultured in a humidified incubator at 37 ° C with 5% CO2 until reaching confluence. Passages were performed as needed for experimental purposes.

Flow cytometry

For immunophenotyping experiments, blood was collected via bleeding from the retro-orbital plexus using a capillary tube (Fisher) while mice were anesthetized with isoflurane (DAR). Bone marrow cells were collected isolated by flushing the femur with RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. Collected blood samples and bone marrow cells underwent erythrocyte lysis using a red blood cell lysis buffer (BioLegend). Following lysis, the remaining white blood cells were washed with phosphate-buffered saline (PBS) and incubated with fluorochrome-conjugated antibodies targeting cell surface markers associated with adaptive and innate immune cells. These markers included CD45 (BioLegend, 103116) CD3 (BioLegend, 100306), CD19 (BioLegend, 152410), CD4 (BioLegend, 100406) CD8

(BioLegend, 126609), CD11c (BioLegend, 117318), Siglec-F (BD, 562068), CD11b (eBioscience, 48-0112-80), Ly-6G (BioLegend, 127613). To measure cell death, propidium iodide (PI) was added into the cell suspension. Stained cells were analyzed using a BD FACS Celesta flow cytometer.

Isolation of neutrophil

Carefully layer the collected blood and bone marrow cells onto a discontinuous Histopaque (Sigma) gradient (3 ml of 1077 on top of the 3ml of 1119). Following centrifugation for 30 min at 700 × g, room temperature, without using a brake, neutrophils were collected from the interface of the Histopaque 1077 and 1119 layers and transfer to 15-ml tubes. Isolated cells underwent thorough washing and resuspended in a RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. The purity was confirmed by flow cytometry neutrophil marker CD11b and Ly6G.

Calcium influx assay

Calcium influx assay was performed as reported previously(23). Briefly, neutrophil was loaded with calcium sensitive dye (BD, 640176) and incubated for one hour at 37˚C. Subsequently, cells were washed once, resuspended in 1 ml of the medium, and kept on ice until analysis. The cells were then stimulated with 1μM of Tg (abcam, ab120286) or 1μM fMLP (sigma, F3506), and intracellular calcium level was monitored by flow cytometry analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-1β and IL-6 concentration in the cell supernatant were quantified using species-specific ELISA kits following the manufacturer's instructions (BD, 559603 and 555240 respectively). For data analysis, a curve fit was applied to the standards, and the sample concentrations were extrapolated from the standard curve.

Phagocytosis assay

To investigate the phagocytic activity of neutrophil under stimulation, fluorescently-labelled latex beads were used in phagocytosis assays. The assay protocol was developed following the manufacturer instructions (Cayman Chemicals, 500290). In brief, neutrophils were resuspended in 200μl phagocytosis buffer to which FITC-labeled beads (1:100) were added and incubated for 2 h at 37℃. The extent of phagocytosis was determined using BZ-X710 series microscope (Keyence) and Filtermax F5 plate reader (Molecular Devices).

GST pull-down assay and mass spectrometry

HEK293T lysates were incubated with GST or GST fused Cisd2 purified from E. coli strain BL21 along with GST4B beads (GE Healthcare), or lysate of HEK293T expressing GST or GST fused Cisd2 were incubated with GST4B beads in binding buffer (20 mM Hepes [pH 7.4], 100 mM NaCl, 1% Nonidet P-40, protease inhibitors) at 4 ˚C for 2 h. GST4B beads were then washed four times with binding buffer, and the proteins associated with the beads were analyzed by SDS/PAGE and subjected to silver staining, Western blot assay. Protein bands were excised and sent to Taplin Biological Mass Spectrometry Facility at Harvard Medical School (HMS) for protein identification.

Immunoprecipitation and Western Blotting

Following a 48-hour incubation post polyethylenimine (PEI) transfection, cells were harvested and lysed in RIPA buffer (500 mM Tris·HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 1 mM EDTA, protease inhibitor). Cell extracts were precleared with protein A/G agarose (Thermo Fisher) for 1 h at 4 ˚C, followed by a 4-hour incubation at 4 ˚C with anti-V5 (Invitrogen, MA5-15253) and subsequent incubation with protein A/G agarose for an additional hour at 4 ˚C. Immunoprecipitates were washed with RIPA lysis buffer and resuspended in SDS sample buffer, boiled at 95 ˚C, resolved on SDS/PAGE gels, and transferred onto polyvinylidene difluoride (PVDF) membranes. Antibody concentrations were as follows: anti-calnexin (Enzo, ADI-SPA-860-F), 1:1,000; anti-GST (Santa Cruz, sc-138), 1:1,000; anti-SERCA (Cell signaling, #9580), 1:1000; anti-β-actin (Santa Cruz, sc-47778), 1:1,000; anti-GFP (Santa Cruz, sc-9996), 1:1,000; anti-BIP (Proteintech, 11587-1-AP), 1:2000; anti-

CHOP (Proteintech, 15204-1-A), 1:2000; and secondary antibodies, affinity-purified with horseradish peroxidase conjugate, 1:5,000. Images were developed with ECL reagent (Thermo Scientific) and imaged on a Bio-Rad ChemiDoc-Touch.

Immunofluorescence analysis

HeLa cells were transfected with plasmid expressing GFP-Cisd2. Then, 24 h after transfection, cells were replated onto coverslips and cultured for another 24 h. Subsequently, the cells were treated with thapsigargin (1 μM) or tunicamyin (2 μg/ml) for 4 h. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.05% saponin and then treated with anti-calnexin. Cells were imaged with laser-scanning confocal microscopy (Nikon Eclipse C1).

Size-exclusion chromatography (SEC)

Size-exclusion chromatography was performed as previously described(24). Briefly, MEFs were lysed in lysis buffer (20 mM Hepes [pH 7.4], 100 mM NaCl, 1% Nonidet P-40, protease inhibitors). The lysate was centrifuged at 10,000 × g for 10 min, and the supernatant was passed through a pre-equilibrated Superose 6 10/300 GL size-exclusion column (GE) with a Biologic Duo Flow system (Bio-Rad). Each fraction was separated by SDS-PAGE, followed by Western blot assay.

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