METHODS

Reagents. Purified nucleotides, propidium iodide, anti-Flag HRP (A8592), anti-Flag agarose beads (A2220), protein G, carbenoxolone, probenecid, flufenamic acid and 18-alpha-glycyrrhetinic acid were obtained from Sigma-Aldrich. YO-PRO-1 and TO-PRO-3 were obtained from Invitrogen. CellTiter-Glo and Caspase-Glo 3/7 were obtained from Promega. Control siRNA (AM4611) and *PANX1* siRNA (ID 134470; sense: 5′-GCAUCAAAUCAGGGAUCCUtt-3′) were obtained from Ambion. Anti-ERK2 (sc-154), anti-Caspase-3 (sc-7148), and anti-GFP HRP (sc-9996) antibodies were obtained from Santa Cruz Biotechnology. Annexin V-FITC and Annexin V-APC were obtained from BD and eBioscience, respectively. cDNA encoding full-length human PANX1 (426 amino acids) was acquired from Open Biosystems (catalogue number MHS1010-58324, accession number BC016931). Anti-hPANX1 serum, affinity purified anti-mPanx1, affinity purified anti-mPanx1 EL2-247 antibodies have been described previously[21,30]. Purified active caspases have also been described previously[12]. Other reagents were obtained as follows: z-VAD(OMe)-fmk (Enzo Life Sciences), anti-Fas (CH11 anti-human clone, Millipore; Jo2 anti-mouse clone, Becton Dickinson), recombinant apyrase (New England Biolabs). mBlueberry2 was provided by R. E. Campbell[31].

Induction of apoptosis. Jurkat T cells (E6.1) resuspended at 2×10^6 cells ml^{-1} in RPMI, 1% BSA, 10 mM HEPES and penicillin/streptomycin/L-glutamine were treated with 250 ng ml^{-1} anti-Fas (activating monoclonal IgM; CH11 clone) or 100 mJ cm^{-2} ultraviolet C irradiation (Stratalinker), and grown at 37 °C, 5% CO_2. Primary murine thymocytes were isolated and treated with cross-linked anti-Fas (5 μg ml^{-1} anti-Fas (Jo2 clone) + 2 μg ml^{-1} protein G) and grown at 37 °C, 5% CO_2. Where indicated, cells were co-treated with zVAD(OMe)-fmk (50 μM or 100 μM) or CBX (500 μM). The induction of apoptosis was performed in medium containing calcium, which can block connexin hemichannels[11].

Preparation of cell supernatants and nucleotide measurement. Cells were incubated for indicated times after receiving either control treatment, or treatment with anti-Fas or UV-irradiation. Cell suspension was then centrifuged at 425*g* for 2 min at room temperature. Supernatants were transferred to a fresh tube and the centrifugation repeated. Supernatants from the second centrifugation were used for nucleotide measurements. ATP was measured using a luciferase/luciferin assay (CellTiter-Glo; Promega) according to manufacturer's instructions, via a 1450 Microbeta TriLux luminometer (Perkin Elmer). UTP concentrations were quantified by the UDP-glucose pyrophosphorylase-based reaction, as previously described[29]. Briefly, 100 μl samples were incubated in the presence of 0.5 U ml^{-1} UDP-glucose pyrophosphorylase, 0.5 U ml^{-1} inorganic pyrophosphatase, 1.6 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES) (pH 7.4), and ~100,000 c.p.m. 1 μM [^{14}C]glucose-1P. Incubations lasted 1 h at 30 °C. Reactions were terminated by heating the samples at 95 °C for 2 min. Conversion of [^{14}C]glucose-1P to [^{14}C]UTP was determined by high-performance liquid chromatography.

Apoptotic caspase activity. Caspase activity assays were performed using the DEVD-based synthetic substrate Caspase-Glo 3/7 (Promega) according to manufacturer's instructions.

Quantitative PCR. cDNA was synthesized from 50 ng of RNA isolated from Jurkat cells (RNeasy, Qiagen) using Superscript III (Invitrogen). qPCR was performed on the ABI StepOnePlus instrument using TaqMan probes (Applied Biosystems). Human pannexin mRNA levels shown are normalized to hypoxanthine phosphoribosyltransferase (*HPRT1*) levels. Primers used are as follows: hPANX1: Hs00209790_m1, hPANX2: Hs00364525_m1, hPANX3: Hs01573439_m1.

Migration assay. Transwell migration assays were performed by applying 100 μl of THP-1 cells at 2×10^6 cells ml^{-1} to the upper chamber (insert) in the same culture medium as the chemoattractant in lower chamber (500 μl) of a 24-well plate with 5-μm pore size Transwells (Corning) for 1 h at 37 °C, 5% CO_2. Supernatants were diluted in some cases to bring the chemotactic activity into the linear range of the migration assay, with the same dilution applied to all samples in each experiment. The percentage of cells that migrated to the lower chamber was determined by flow cytometry using 5.1 μm AccuCount beads (Spherotech) and calculated as the percentage of input cells. Where indicated, 0.05 U ml^{-1} recombinant apyrase was added to chemoattractant for 5 min at room temperature.

Air pouch studies. All animal studies were performed in accordance with the University of Virginia Animal Care and Use Committee guidelines. Animals were housed in a specific pathogen-free facility. Female C57BL/6 mice were used (Charles River Laboratories). Air pouch experiments were performed as described previously[32], using mice aged 8–12 weeks. In brief, 5 ml of 0.2 μm-filtered air was injected subcutaneously into the mid-dorsal region. After 3 days, the same pouches were injected with 3 ml of 0.2 μm-filtered air to maintain the pouch. Four days later the pouches were injected with 1 ml of 0.2 μm-filtered supernatants from control or ultraviolet-treated Jurkat cells. Supernatants were produced in RPMI with 5% FBS, 10 mM HEPES, and penicillin/streptomycin/L-glutamine. After 24 h, cells from the air pouch were collected by two lavages of 2 ml of HBSS/1% FBS. Collected cells were resuspended in equal volumes and cell counting was performed by flow cytometry using quantification beads (Spherotech). For analyses of specific populations in the air pouch, cells were treated with anti-CD16/32 for 15 min on ice to block Fc receptor binding, followed by addition of the indicated fluorescently labelled antibodies for 30 min on ice. After washing, the cells were analysed with a FACS Canto instrument (Becton Dickinson).

PANX1 constructs. Human PANX1–Flag construct was generated by PCR cloning of *PANX1* cDNA (Open Biosystems; catalogue number MHS1010-58324) into pEBB-Flag vector. Mutations were performed using QuikChange mutagenesis kit (Stratagene). Fidelity of all constructs was confirmed by sequencing. Site B (DVVD) was C-terminal to and continuous with another predicted caspase cleavage site (IKMD); therefore, both aspartic acid residues were mutated to alanine to ensure complete disruption of the cleavage region (IKMDVVD mutated to IKMAVVA). Glutamic acid substitutions were also performed, in order to conserve charge at the cleavage site (IKMDVVD mutated to IKMEVVE). PANX1 truncation mutant (amino acids 1–371) was produced by PCR to end at amino acid 371, immediately followed by a Flag tag (8 amino acids: DYKDDDDK). This resulted in a 379 amino acid truncation mutant protein, which is exactly the same size as would be produced by caspase cleavage after the last aspartic acid residue in site B.

siRNA, transient, and stable transfections. Transfections were carried out using the BTX Square Pulse T820 electroporator with 5–10 million Jurkat T cells per transfection. For siRNA transfection, cells were transfected with 200 nM siRNA (Ambion) and used 72 h after transfection. For transient DNA transfections, Jurkat cells were electroporated with 5 μg of control vector or PANX1–Flag construct along with 5 μg mCherry expression construct (marker), and used 48 h after transfection. For generation of stable lines, Jurkat cells were electroporated with 10 μg of plasmid (pEBB PANX1–Flag) along with a pA-puro vector, and cultured in 1 μg ml^{-1} puromycin before and during limiting dilutions to isolate single-cell clones. Puromycin-resistant clones were screened for expression of Flag by immunoblotting.

Measuring selective membrane permeability during apoptosis. Jurkat cells were induced to undergo apoptosis (as described above) in media containing 1 μM YO-PRO-1 or TO-PRO-3 dyes. Cells were washed in PBS and resuspended in either PBS/0.5% BSA or binding buffer for annexin V staining. Dye uptake and cell surface staining was assessed by flow cytometry.

Patch-clamp analysis. Whole-cell recordings were obtained at room temperature using 3- to 5-MΩ borosilicate glass patch pipettes and an Axiopatch 200A amplifier (Axon Instruments) in a bath solution composed of 140 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose (pH 7.3). Internal solution contained 30 mM TEACl, 100 mM $CsMeSO_4$, 4 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM HEPES, 10 mM EGTA, 3 mM ATP-Mg and 0.3 mM GTP-Tris (pH 7.3). Ramp voltage clamp commands were applied at 5-s intervals using PCLAMP software and a Digidata1322A digitizer (Axon instruments). Whole-cell peak conductance was taken at +90 mV and normalized to cell capacitance. CBX-sensitive current was taken as the difference in peak conductance before and after CBX application relative to the initial peak current.

Immunoblotting. Samples were analysed by SDS–PAGE and immunoblotting using the following antibody concentrations/dilutions: anti-human PANX1 serum (1:5,000), affinity-purified anti-Panx1 EL2-247 (0.2 μg ml^{-1}), affinity purified anti-murine Panx1 (0.2 μg ml^{-1}), anti-ERK2 (1:10,000) and anti-Caspase-3 (1:1,000).

Purified PANX1 proteins. HEK 293T cells were transfected with Flag-tagged ELMO1 or PANX1 plasmids using calcium phosphate transfection (ProFection, Promega). After 48 h, whole-cell lysates were immunoprecipitated using anti-Flag beads, washed in wash buffer, and resuspended in TBST + 10% glycerol before use in *in vitro* cleavage reactions.

***In vitro* caspase cleavage assay.** Active recombinant caspases were produced and characterized as described previously[12]. *In vitro* cleavage reactions were performed in the following reaction buffer: 100 mM NaCl, 10% sucrose, 0.1% CHAPS, 20 mM PIPES, 1 mM EDTA and 10 mM dithiothreitol. Immunoprecipitated Flag-tagged proteins were resuspended in reaction buffer, and purified active caspase was added to a final concentration of 100 nM. The reaction was carried out for 1 h at 37 °C and terminated by addition of SDS sample buffer and boiling for 5 min. The reactions were analysed by SDS–PAGE and immunoblotting.

Scrape assay. Scrape assays were performed as originally described[33]. Briefly, confluent monolayers of HeLa cells or HeLa cells stably expressing Connexin 43 (Cx43) were scraped with a blunted 22-gauge needle in PBS containing 1 mg ml^{-1} Lucifer yellow. Five minutes after scrape, cells were washed with PBS, fixed with 4% paraformaldehyde and viewed on an Olympus IX70 microscope.

Phagocytosis assay. Bone marrow-derived macrophages (BMDMs) were prepared as described previously[32]. For phagocytosis assays, BMDMs were plated at a density of 100,000 cells in a 24-well plate in RPMI+10% serum overnight, and

stained for 5 min with 1 mM CFSE-SE (Invitrogen) before the engulfment assay. For apoptotic targets, Jurkat cells were induced to undergo apoptosis using anti-Fas treatment as described above in the presence or absence of 500 μM CBX, then stained with TAMRA-SE (Invitrogen; to determine gross binding/uptake of apoptotic cells) or Cypher-5E (GE Health; a pH-sensitive dye that displays enhanced fluorescence upon acidification of the apoptotic cell in the phagosome, to monitor internalization). BMDMs were then incubated with 2 million apoptotic Jurkat cells (in the presence or absence of 500 μM CBX) for ~1 h, trypsinized and analysed on a BD FACSCanto flow cytometer. The collected flow cytometry data were analysed using FlowJo software.

Statistical analyses. Data are presented as the mean ± standard error of the mean (s.e.m.) or mean ± standard deviation (s.d.), unless noted otherwise. Statistical significance for comparisons was determined by the Student's two-tailed *t*-test, unless noted otherwise. A *P* value less than 0.05 was considered statistically significant.

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