RTP801 is required for ceramide-induced cell-specific death in the murine lung

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Online Data Supplement

Intratracheal instillations. For the intra-tracheal instillation of ceramide C12:0, the lipid was first solublilized in ethanol, and then suspended in sterile perfluorocarbon (15μl). The oxygen-carrying properties of perfluorocarbon ensure adequate tolerance by the animal at these volumes, while its physical-chemical properties allow for efficient distal lung delivery following intra-tracheal instillation (11, 13). Mice were anesthetized by brief inhalational halothane exposure, the tongue was gently pulled forward by forceps and the trachea instilled with ceramide-containing or vehicle (ethanol) containing perfluorocarbon solution applied at the base of the tongue *via* a blunt angiocatheter (25). A similar procedure was followed for the instillation of *rtp*801 expression plasmid DNA or an empty plasmid DNA (50μg) which was delivered intra-tracheally in 30 μl of saline and 70 μl perfluorocarbon. For the intra-tracheal delivery of ceramide C16:0 (conjugated with either polyethylene glycol 2000 and resuspended in saline), the trachea was exposed in aseptic conditions following anesthesia and either the compound or just the vehicle were introduced directly into the trachea using a needle. Following suture of the neck soft tissues, mice were allowed to recover.

Measurement of lung function is detailed in the Methods Online Supplement. Mice (RTP801-null or wild type littermates) were anesthetized with inhaled isoflurane and suspended vertically from their incisors. The neck was transilluminated with a light source and the glottis opening was visualized with a custom-made laryngoscope blade. A 0.025” diameter guide wire was probed through the glottis opening, and a 20-gauge Teflon catheter was advanced over the guide wire into the trachea. Following removal of the wire, the animal was mechanically ventilated with a rodent ventilator using room air,

at a rate of 140 breaths per minute, a tidal volume of 0.3 ml, and 2 cmH_2O of positive end-expiratory pressure. The animals were placed on a heated (37C^o) pad and pulmonary function tests were then performed with the Flexi Vent system (Scireq, Montreal, PQ, Canada). At the conclusion of testing, isoflurane was discontinued, and the animal was allowed to emerge from anesthesia. Once spontaneous respiration had been resumed and reflexes had been returned, the tracheal cannula was removed. Subsequently, mice were euthanized and their lungs were processed as described below.

Bronchoalveolar lavage (BAL)**.** The BAL fluid was collected by lavaging the lungs with aliquots totaling 3 ml of Ca^{2+}- and Mg^{2+} -free PBS supplemented with 0.1 mM EDTA. Samples were centrifuged (6 min; 500g; 4°C). Cell pellets were collected in 1 ml of red blood cells (RBCs) lysis buffer and following washing; cells were resuspended in PBS and counted in hemocytometer. Cytospin slides containing 10,000 cells were made with total volume 350 μl of PBS, centrifuged at 1350 rpm for 5 min and stained using 3 step stain set (Richard Allen Scientific). The slides were rinsed in water twice and allowed to dry, then cover glasses were applied with mounting medium (Fisher Scientific) and cells were scored by a technician blinded to the identity of the experimental group.

Lung tissue harvesting. A cannula was inserted into the trachea and lungs were flushed by perfusing 20 ml of PBS through the right ventricle. The right bronchus was ligated. A pre-warmed solution of low melting point agarose (0.25% agarose; 10% formalin; PBS) was introduced into the left lung under constant pressure (20 cm H_2O). The trachea was clamped, the lungs and the heart were dissected *en block*, transferred into 50 ml conical

tube, and kept at 4^{o}C for 5-10 min. The right lung was aliquoted and snap frozen in liquid N_2 and the left lung was transversally sectioned in the coronal plane, transferred into a plastic fixation cassette and then paraffin embedded.

Lung tissue dissociation in single cell suspension. Mice were euthanized with isoflurane and cannulated with an intra-tracheal catheter. Lungs were perfused via the right ventricle with Ca^{2+}- and Mg^{2+} -free PBS, at 37C^{o}, until they blanched. Large vessels and bronchi were discarded. A quarter of each lung was reserved for biochemical assays and snap-frozen in liquid nitrogen. The remaining lung was placed in 60 mm tissue culture dishes containing dissociation solution and then dissected and minced into 1-2 mm diameter fragments, followed by immediate transfer into 50 ml conical tube. The lung tissue dissociation solution contained fetal bovine serum (10%) in Dulbecco's modified Eagle medium, DNase I (6.5 µg/ml), and collagenase I (12 µg/ml; Roche, Indianapolis, IN). Lung fragments were disintegrated in this solution (30 min; 37°C) until a clear cell suspension was obtained. Cell suspension was filtered through a cell strainer (70 µm; Fisher Scientific, Fair Lawn, NJ), followed by centrifugation (5 min, 500g, 4^{o}C). Cell pellets were resuspended in PBS, followed by the addition of Geyes solution (erythrocyte cell lysis buffer) and subjected to repeated centrifugation. Cell pellets were then resuspended in 1ml of PBS with 1% FBS and used as needed.

Apoptosis was measured in whole lung homogenates, in specific cell populations isolated following lung tissue dissociation in single cell suspensions, or on fixed lung sections, as follows. *Caspase-3 activity in lung homogenates* was measured using a fluorometric

assay (Apo-ONE; Promega, Fitchburg, WI) as described in (9). Assessment of apoptosis in specific lung cell populations was performed in cell suspensions isolated by lung dissociation as described above, followed by *detection of cleaved caspase-3 using flow cytometry.* For channel setup Jurkat cells (Lymphoma T cells from ATCC; 10^6 in 100ml) stained with FITC labeled anti CD3 (eBioscience) for FL1 and with Phycoerythrin (PE) labeled anti CD3 (eBioscience) for FL2. Cell suspensions were incubated with anti CD32/16 antibody (Santa Cruz Biotechnology; 1μg/10^6 cells; 10 min), followed by washing and transfer into flow cytometry tubes. Labeling of selected cell populations was achieved with rat anti-mouse CD31 FITC conjugated antibodies (BD Pharmingen; 30 min) for the detection of endothelial cells, using a rat FITC-conjugated IgG 2 (eBiosciences) as isotype control. For the detection of type I pneumocytes, Alexa Fluor 488 labeled Golden Syrian Hamster anti-Podoplanin IgG antibody (eBiosciences) was used, with Golden Syrian Hamster FITC-conjugated (eBiosciences) IgG as isotype control. Cell suspensions stained for alveolar type II cell markers were first prepared for intracellular staining. All samples underwent fixation and permeabilization with paraformaldehyde (1% in PBS) and Triton-X 100 (0.1% in PBS), respectively, and stained with pro-surfactant C polyclonal rabbit anti-mouse (Millipore/Abcam) FITC-labeled antibodies. Antibody labeling with FITC was achieved using a specific antibody labeling kit (Pierce) following the manufacturer's instructions. As isotype control, cells were stained with similarly labeled rabbit anti-mouse polyclonal non-specific IgG (SouthernBiotech). Staining for active caspase-3 in lung cell suspensions was achieved by using PE-conjugated affinity purified polyclonal rabbit antibody raised against anti-active caspase-3 (BD Pharmingen), and an isotype control PE-conjugated non-specific

IgG antibody (SouthernBiotech). Following staining (30 min), samples were washed, centrifuged (500g; 5 min; room temperature) and cell pellets were suspended in BSA (1% in PBS) for flow analysis. Apoptotic UV-irradiated Jurkat cells were used as a positive control for active caspase-3 staining. *Active caspase-3 immunochemistry* was performed on lung sections as previously described (18). Briefly, following deparaffinization and hydration, sections were blocked with goat serum (10%) and incubated with anti-active caspase-3 antibody (Cell Signaling; 1h at room temperature or overnight at 4^{o}C). Slides were then stained with biotin-conjugated goat anti-rat IgG secondary antibody (1:100; Vector Laboratories, Burlingame, CA) and streptavidin-coupled phycoerythrin or fluorescein isothiocyanate (1:1,000; Vector). Sections were counterstained with DAPI and mounted with Mowiol 488 (Calbiochem). Microscopy was performed on either Nikon Eclipse (TE200S) inverted fluorescence or a combined confocal/ multiphoton (Spectraphysics laser, BioRad MRC-1024-MP) inverted system. Images were captured in a masked fashion and quantitative data were obtained by Metamorph Imaging software (Molecular Devices, Sunnyvale, CA) as previously described (18).

Detection of RTP801 by immunohistochemistry was performed after deparaffinization and antigen retrieval with citrate solution (pH 6.0; 30 min). After inhibition of endogenous peroxidase activity by incubation in H2O2 (3% sin methanol; 30 min) slides were blocked in goat serum (10 % in TBS, 30 min). Primary antibody against RTP801 was applied (REDD1, rabbit anti-mouse, Proteintech cat. No 10638-1-AP; 1:200; 1h), followed by treatment with secondary biotinylated goat anti rabbit antibody (1:200; 1h), streptavidin peroxidase (30 min), and chromogen (DAB; 5 min). A set of sections was

then counterstained with Mayor's hematoxylin. Slides were then coded and images of the lung parenchyma were captured in an unbiased fashion, using the 40x objective, excluding areas with large or medium sized airways or vessels. The intensity of RTP expression was then quantified on coded images, using a macro developed for Metamorph, which integrates intensity (in pixels) and area (in pixels) of the positive immunohistochemistry staining, generating arbitrary units. Following decoding, data are analyzed by comparison among experimental groups.

Lung histology and morphometry. Four micrometer paraffin sections on slides were deparaffinized in Clear-Rite 3 (Richard–Allan Scientific), followed by hydration and staining with hematoxylin and eosin.

Western blotting. Lung tissue was homogenized in a lysis buffer containing HEPES (20 mM, pH 7.5), $MgCl_2$ (1.5 mM), NaCl (150 mM), glycerol (10%), Triton X- 100 (1%), EDTA (2 mM), Na_3VO_4 (2mM), NaF (50 mM), PMSF (1 mM) and Protease Inhibitor Cocktail Set I (Calbiochem). Protein lysates were electrophoresed through a SDS- PAGE and transferred to nitrocellulose membrane using the Criterion system (Biorad). The membranes were blocked with Superblock blocking buffer (Pierce Biotech.) and incubated overnight with rabbit anti-mouse RTP801 raised at Quark Pharmaceuticals Inc, and with secondary anti-rabbit antibodies conjugated with horseradish peroxidase (Vector). The blots were developed using the enhanced chemilluminescence kit (GE Healthcare).

Statistical analysis was performed with Sigma Stat (Systat Software Inc, Chicago, IL, USA), using unpaired Student t-test, ANOVA, or Kruskal-Wallis one-way ANOVA on ranks for morphometry analysis. Statistical difference was accepted at $p<0.05$. Numerical data are presented either in bar graphs showing means and standard error of the means or in box plots: the horizontal lines within the boxes show medians; bounds of the boxes show 25^{th} and the 75^{th} percentiles of the data, respectively; and whiskers show outliers, if applicable (5^{th} and 95^{th} percentiles, respectively).