

Supplementary Information for

ISM1 protects lung homeostasis via cell surface GRP78-mediated alveolar macrophage apoptosis

Authors

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SI Materials and Methods

Reagents. Antibodies used for western blot: anti-MMP-12 (ab52897, Abcam), anti-MMP-9 (ab38898, Abcam), anti-p65 (10745-1-AP, Proteintech), anti- β -Actin Antibody (C4, Santa Cruz Biotechnology), anti-TGF- β 1 (V, Santa Cruz Biotechnology), anti-VEGF-A (A-20, Santa Cruz Biotechnology), anti-Neutrophil Elastase (ab68672, Abcam), anti-Alpha-1-Antitrypsin (16382-1-AP, Proteintech). Primary antibodies used for immunohistochemistry: anti-MMP-12 (ab66157, Abcam), anti-MMP-9 (ab38898, Abcam), anti-TGF- β 1 (V, Santa Cruz Biotechnology), anti-VEGF-A (A-20, Santa Cruz Biotechnology), anti-ISM1 (for mouse lung: E-20, Santa Cruz Biotechnology; for human lung: custom antibody 3M8, AbMart), anti-GRP78 (A-10, Santa Cruz Biotechnology), anti-His-probe (H-15, Santa Cruz Biotechnology), anti-Cleaved caspase-3 (Asp175, Cell Signaling Technology). Primary antibodies used for immunofluorescence: anti-ISM1 (E-20, Santa Cruz Biotechnology), anti-p65 (10745-1-AP, Proteintech), anti-CD68 (M-20, Santa Cruz Biotechnology), anti-6X His tag antibody (ab9136, Abcam), anti-GRP78 (A-10, Santa Cruz Biotechnology), anti-Cleaved caspase-3 (Asp175, Cell Signaling Technology), anti-SP-C (FL-197, Santa Cruz Biotechnology), anti-PCNA (PC10, Santa Cruz Biotechnology), anti-GRP78 (A-10, Santa Cruz Biotechnology), Neutrophil Marker (NIMP-R14, Santa Cruz Biotechnology), anti-Endomucin (V.7C7, Santa Cruz Biotechnology), anti-Occludin (H-279, Santa Cruz Biotechnology), anti-Aquaporin 5 (ab78486, Abcam). Antibodies used for flow cytometry were purchased from Miltenyi Biotec: CD45-VioBlue (Clone: REA737), Anti-Siglec-F-APC (Clone: REA798), CD11c-PE (Clone: REA754), CD11b-VioBright FITC (Clone: REA592) and Anti-Ly-6C-PerCP-Vio700 (Clone: REA796). ISM1 was measured in mouse lung lysates using LEGEND MAX™ Mouse Isthmin ELISA Kit (438907, BioLegend). Reactive oxygen species were measured using OxiSelect™ In Vitro ROS/RNS Assay (STA-347, Cell Biolabs). Cell proliferation was measured using Click-iT™ EdU Proliferation Assay for Microplates (Invitrogen, C10499). rISM1 was produced as previously described (1). Liposome-encapsulated clodronate was purchased from Liposoma. MitoTracker™ Red CMXRos (Invitrogen, M7512) was used to label mitochondria. siRNA-mediated knockdown was carried out using Lipofectamine™ 3000 Transfection Reagent (L3000001). Reagents were used according to manufacturer's instructions.

Mice. All animal experiments were conducted in accordance with approved protocols by the National University of Singapore, Institutional Animal Care and Use Committee (IACUC protocols BR15-1100 and R18-0588). All wild-type mice were purchased from InVivos Pte Ltd, Singapore. *Ism1*^{-/-} mice (FVB/NTac and C57BL/6J) were in-house generated using pronuclear microinjection of recombinant Cas9 and guide RNAs targeting 5'-ctgcacatcaccggttctgcgccc-3' (gRNA1, PAM sequence underlined) and 5'-gcggatccggagcctccgaccgg-3' (gRNA2, PAM sequence underlined) of *Ism1* exon1. Filial generations of *Ism1*^{-/-} mice were identified via genotyping primer pairs (FVB/NTac: 5'-cagtcctgggattgtcctcg-3' and 5'-ccttctgcaatgtaccaagctct-3', C57BL/6J: 5'-cgcgcgactcaagaggatgg-3' and 5'-actgggaccgctgacgttg-3') and sequencing, before being selected for subsequent breeding and colony maintenance. All mice were housed under standard 12-hour light-dark cycle, with food and water available ad libitum. Mice were anesthetized with isoflurane prior to all intratracheal instillations.

Mouse lung histology and imaging. Mouse lungs from respective experiments were inflated and fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned in 5 μ m thickness. Histology and pathology scoring for emphysema in FVB/NTac WT and *Ism1*^{-/-} mice were performed by a veterinarian pathologist on hematoxylin and eosin (H&E) stained lungs. Mean linear intercepts (MLI) and radial alveolar counts were quantified as described with ten to fifteen fields analyzed per mice (2, 3). Bronchial epithelial cell counts and measurements were quantified using ImageJ software (NIH). For IF and IHC staining, lung sections were subjected to antigen retrieval by pressure-cooking in sodium citrate buffer after deparaffinization. Slides were rinsed with PBS before blocked with 3% BSA in PBS for 1 hour. 3% hydrogen peroxide quenching was carried out on lung sections intended for immunohistochemistry. Lung sections were incubated overnight with the respective antibodies in a humidified chamber at room temperature, washed three times with 0.1% PBST, incubated with respective secondary antibodies for 1 hour at room temperature and washed again with three times with 0.1% PBST. IF-stained slides were counterstained with DAPI, and IHC-stained slides were incubated in liquid DAB+ chromogenic substrate (Dako) with hematoxylin counterstain. Lung sections were stained with Periodic Acid-Schiff (87007, Thermo Fisher) and Elastin Stain Kit (ab150667, Abcam). Images were acquired with Zeiss Axiovert 200, Zeiss LSM-510 Meta and Olympus FV1000 confocal microscopes and analyzed using ImageJ software (NIH).

Lung immune cell quantifications. Flow cytometry was carried out using BD LSRFortessa and analyzed on FlowJo software (BD Biosciences). Gating strategy for Siglec-F⁺CD11c⁺ resident alveolar macrophages in [SI Appendix, Fig S3](#). For immunofluorescence staining, AMs and neutrophils were quantified using antibodies for CD68 and Ly6G/6C respectively, or by morphology for cytospin slides. Five random fields were chosen per mouse.

Gelatin zymography. Pre-casted gelatin zymogram gels (Invitrogen Novex) were used to detect endogenous MMP-9 and MMP-2 activity in 2-month old FVB/NTac wild-type and *Ism1*^{-/-} mice. Mouse lungs were homogenized in Tris buffer (20 mM Tris-HCl, pH 7.5), and gel subsequently washed and incubated for 24 hours in 2.5% and 1% Triton X-100-containing Tris buffer with 5 mM CaCl₂, respectively. Gel was stained in Coomassie blue and destained to reveal clear bands, indicative for MMP activity.

Cell culture. MH-S (CRL-2019™) was purchased from ATCC and cultured in RPMI-140 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). Primary AMs were harvested from 2-month old wild-type and *Ism1*^{-/-} FVB/NTac or C57BL/6J mice as described (4), and cultured in RPMI-140 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were maintained at 37 °C in a 5% CO₂ incubator. Plasma membrane fractions of MH-S cells were isolated as previously described (5). RNAi-mediated knockdown of GRP78 in MH-S cells was carried out using published sequences via lipofection for 24 hours (6):

siGRP78-1, 5'-TTCTACCATAAGTGACACCAATAAATGTT-3',

siGRP78-2, 5'-ACCTATTCCTGCGTGGTGTGTTCAAGAA-3',

siCtrl-1 (siGRP78-1 scramble), 5'- ACTGCACTACCTCATAGAAGTATTAATAT-3',

siCtrl-2 (siGRP78-2 scramble), 5'- GTTCTGATGAATCCGATCTCTAGTGGACC-3'.

Cigarette smoke extract (CSE) was prepared by air-pumping the smoke of one 3R4F reference cigarette (University of Kentucky, Lexington, KY, USA) into 10 mL of RPMI-140 culture medium. This was designated as 10% CSE, and sterile-filtered and further diluted to 0.25% CSE with RPMI-140 culture medium for immediate MH-S treatment for 24 hours.

Apoptosis determination. Apoptosis were measured using the IncuCyte ZOOM live cell imaging system (Essen Bioscience). MH-S cells or primary AMs were seeded at a density of 50,000 cells per 96-well and apoptosis was measured hourly using IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent (Cat. No. 4440, Essen Bioscience) according to manufacturer's instruction. MH-S cells were pretreated for 24 hours with 50 nM thapsigargin (Sigma-Aldrich) in RPMI-140 media supplemented with 10% heat-inactivated FBS, and then treated with 1 µM rISM1 with and without anti-GRP78 (A-10, Santa Cruz Biotechnology) antibody neutralization for 16 hours under same culturing conditions. Treatments were carried out in triplicate wells, and 4 images per well were taken for quantifications. Primary AMs were treated with 1 µM rISM1 for 16 hours under same culturing conditions. Experiment groups were carried out in quadruplicate wells, and 4 separate fields per well were taken for quantifications.

Efferocytosis assay. Primary AMs were isolated from 2-month old C57BL/6J wild-type and *Ism1*^{-/-} mice and seeded at a density of 100,000 cells per 96-well. MH-S cells were pre-labeled with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) (Invitrogen) and apoptosis generated by UV irradiation and confirmed via trypan blue uptake. Apoptotic MH-S cells were then overlaid in the primary AM culture in the ratio of 1:10 (apoptotic MH-S to primary AMs) and incubated for 24 hours. Quantifications were carried out using five microscopic fields per mouse, and efferocytosis capacity expressed as a percentage of cells containing fluorescent-labeled apoptotic bodies over total number of cells per field.

ISM1 and GRP78 antibody validation. HEK293FT cells were transfected with a hISM1 overexpressing plasmid (hISM1-OE) or empty vector (EV) via Lipofectamine 3000 following manufacturer's instruction. Cells were trypsinized and collected as a cell pellet 24 hours post-transfection and processed for paraffin embedding and sectioning. Cell pellet sections were subjected to antigen retrieval followed by primary antibody incubation for hISM1 (custom mouse Mab 3M8,

AbMart) or GRP78 (A-10, Santa Cruz Biotechnology). As controls, primary antibodies were pre-adsorbed with recombinant ISM1 or a GRP78 peptide (corresponding to antigen sequence) respectively at a 1:10 antibody-to-antigen weight ratio for 24 hours in 4°C. After secondary antibody incubation, cell pellet sections were then stained with DAB+ chromogenic substrate (Dako) and counter-stained with hematoxylin.

Human lung tissue. The use of human samples was approved by National University of Singapore Institutional Review Board (NUS-IRB Ref No. N-18-057E). Formalin-fixed and paraffin-embedded de-identified lung sections were provided by the Lung Tissue Research Consortium (LTRC), National Institutes of Health (NIH), USA. Expression of hISM1 by immunostainings in non-COPD and COPD patients was blindly graded by two researchers with six to ten random fields were chosen per human lung section. hISM1 expression in AMs derived from IHC staining was based on intensity and graded as 1 (low), medium (2) and high (3); and hISM1 expression in AMs derived from IF was based on frequency of positive hISM1 expression and graded as 1 (<25%), 2 (25 – 70%) and 3 (>70%). Both grades from IHC staining intensity and IF frequency were multiplied to derive final hISM1 expression scores of 1 – 3, 4 – 6 or 9 and assigned as groups +, ++ and +++ respectively. Smokers are defined as individuals who have used at least 100 cigarettes in their lifetimes.

Statistical analysis. Statistical analyses were performed using Prism (Graphpad) Software. Comparisons between two groups were done using two-group two-tailed Student's *t*-test, and multiple group comparisons were done using one-way or two-way ANOVA with Tukey's post hoc test. Multiple linear regression analysis was used to determine the relationship between AM apoptosis and hISM1 expression after adjusting for age, sex, smoking, GOLD scores and GRP78 expression in [SI Appendix, Tables S2](#). Associations between hISM1 expression and COPD or smoking were determined using Chi-square test of trend in [SI Appendix, Tables S3 and S4](#). Correlation between GRP78 expression and AM apoptosis was determined using Pearson correlation. Results are shown as mean \pm SD and sample sizes for each experiment are indicated accordingly on the figures or legend. A *P*-value of < 0.05 was considered significant.

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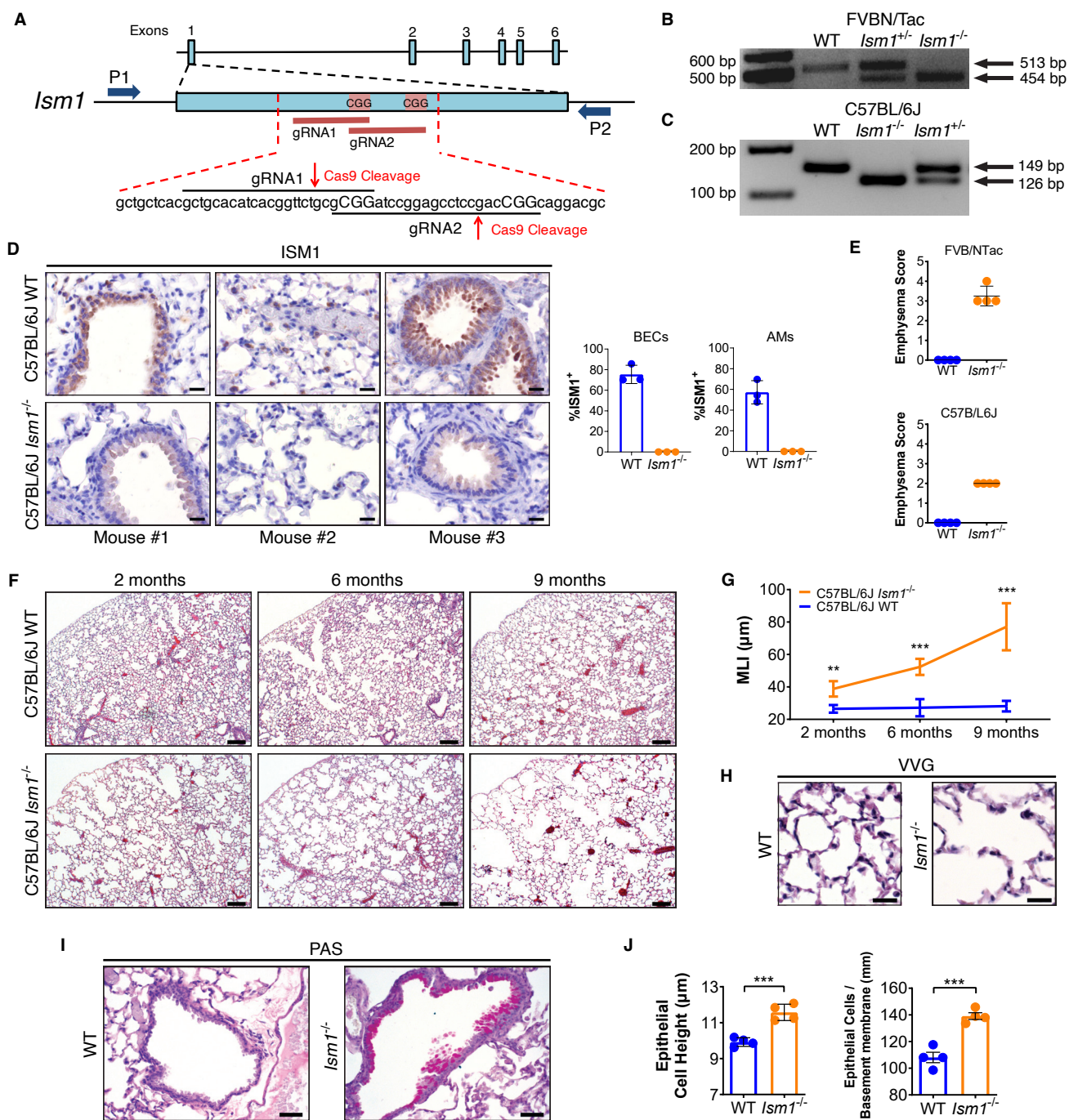


Fig. S1. Generation of *Ism1*^{-/-} mice. (A) Schematic diagram of CRISPR/Cas9 targeting *Ism1* via guide RNA pair gRNA1 and gRNA2. (B) Genotyping PCR of FVB/NTac and (C) C57BL/6J WT, *Ism1*^{+/-} and *Ism1*^{-/-} mice. (D) Representative immunohistochemistry (IHC) staining and quantifications of bronchial epithelial cells (BECs) and alveolar macrophages (AMs) expressing ISM1 in C57BL/6J WT and *Ism1*^{-/-} mice lungs. (E) Pathology grading of emphysema in 2-month old FVB/NTac and C57BL/6J WT and *Ism1*^{-/-} mice. (F) H&E stained left lung lobes and (G) mean linear intercepts (MLI) of C57BL/6J WT and *Ism1*^{-/-} mice at 2, 6 and 9 months of age. (H) Verhoeff-Van Gieson (VVG) stained lungs showing loss of elastin (black), and (I) Periodic Acid-Schiff (PAS) stained airways showing mucus hypersecretion (red) in 2-month old FVB/NTac *Ism1*^{-/-} mice. (J) Measurements of bronchial epithelial cell height and cell number in 2-month old FVB/NTac WT and *Ism1*^{-/-} mice. Data are mean \pm SD and were analyzed by two-group two-tailed Student's *t*-test (G and J). ***P* < 0.01, ****P* < 0.001. *n* = 3 – 4 mice per group. Scale bars: 20 μ m (D, H, I), 200 μ m (F). All figures are independent experiments with *n* = 3 – 4 mice per group.

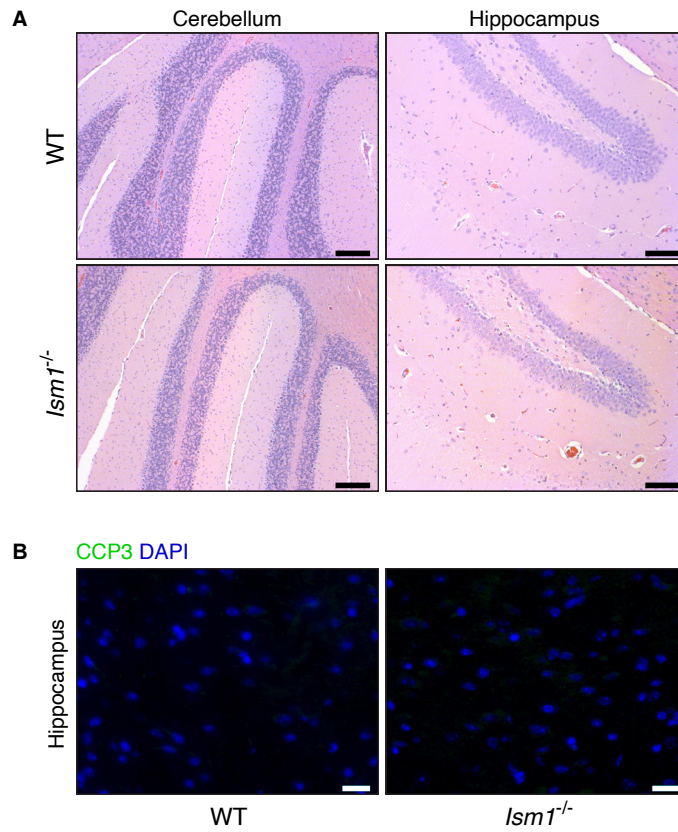


Fig. S2 *Ism1*^{-/-} mice present no gross abnormalities in the brain. (A) H&E and (B) IF staining for cleaved caspase-3 (CCP3, green) showing no gross abnormalities or apoptosis difference in cerebellum and hippocampus of FVB/NTac WT and *Ism1*^{-/-} mice at 2 months of age. Nuclei is stained by DAPI (blue). Scale bars: 200 μ m (A, cerebellum), 100 μ m (A, hippocampus), 50 μ m (B). All figures are from one independent experiment with n = 4 mice per group.

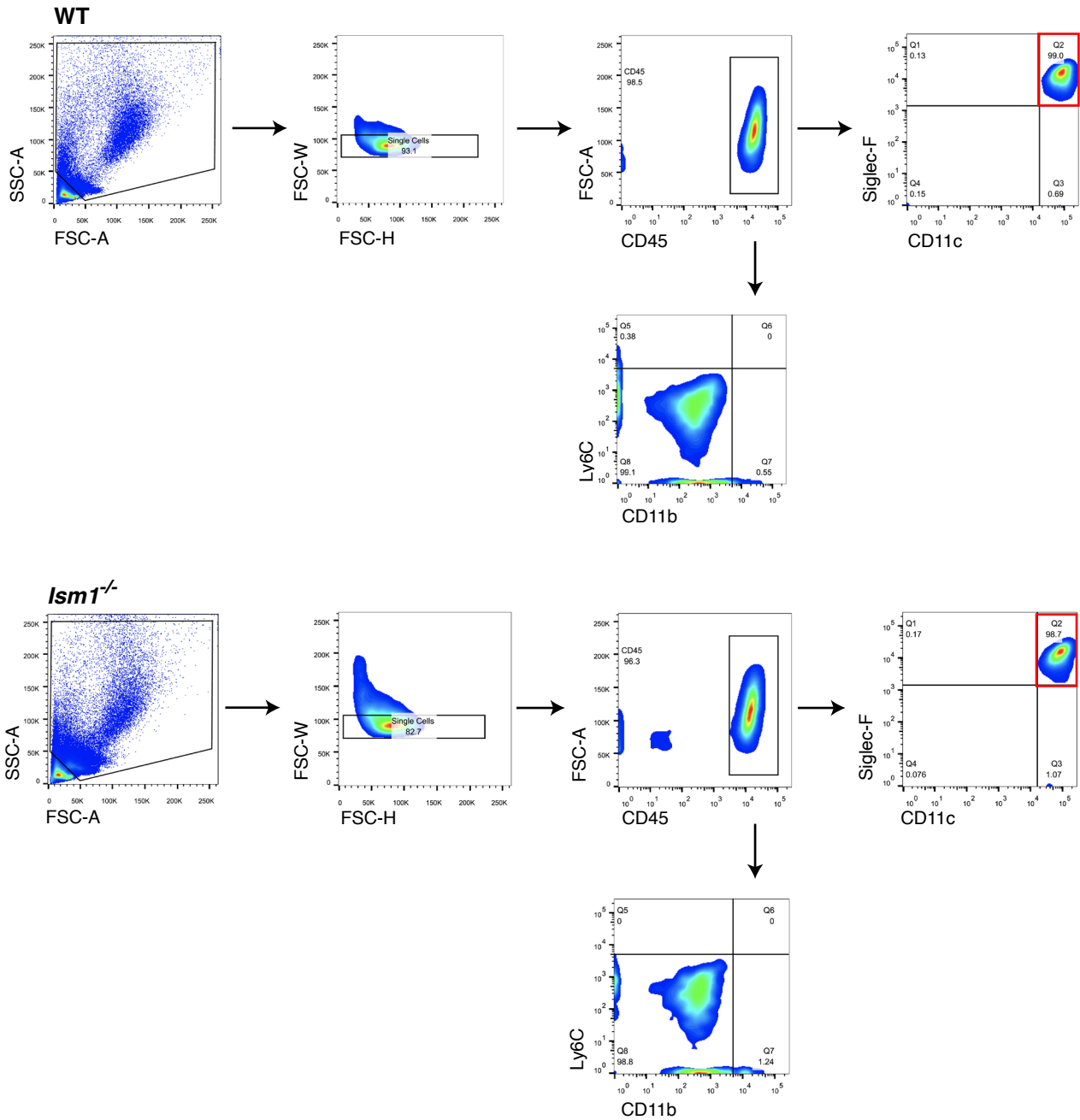


Fig. S3. Gating strategy for flow cytometry analyses of resident AMs. Representative gating strategies for flow cytometric analysis and quantifications for resident AMs are denoted in red rectangle (Siglec-F⁺CD11c⁺Lyc6C⁻CD11b⁻) in bronchioalveolar lavage fluid (BALF) from 2-month old FVB/NTac WT and *Ism1*^{-/-} mice. The AMs identified in this study are all tissue-resident AMs. No CD11b⁺ monocyte derived AMs were detected in BALF.

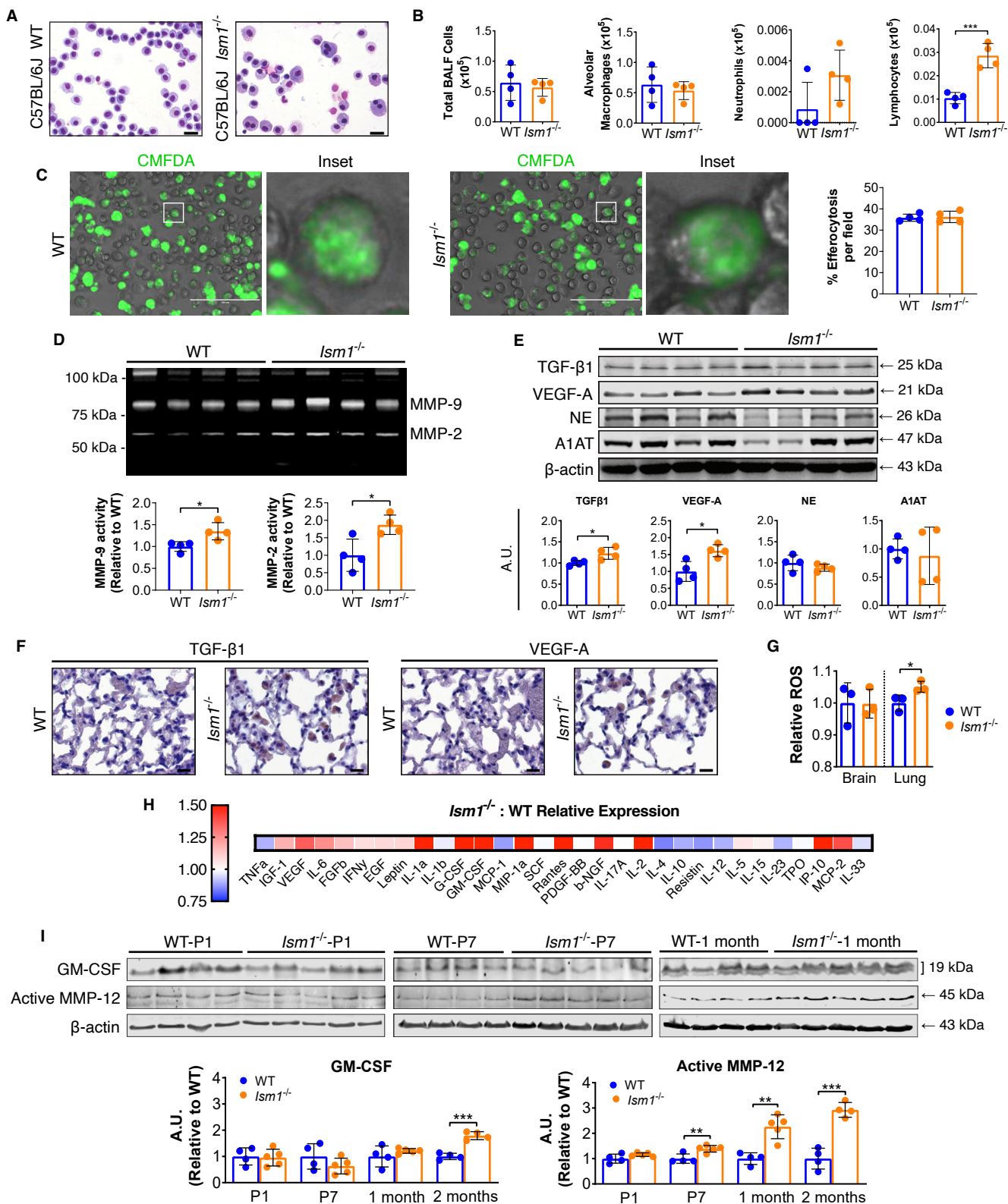


Fig. S4. Characterization of *Ism1*^{-/-} mice. (A) Liu-stained cytopsin and (B) quantifications of BALF cells from 2-month old C57BL/6J WT and *Ism1*^{-/-} lungs. (C) AM efferocytosis capacity is not changed in *Ism1*^{-/-} mice. Representative microscopic images showing efferocytosis of CMFDA-labelled apoptotic MH-S cells (green fluorescent) by primary AMs from 2-month old C57BL/6J WT and *Ism1*^{-/-} mice. Quantification is shown on the right. (D) Increased MMP-9 and MMP-2 activity shown by gelatin zymography using 2-month old FVB/NTac WT and *Ism1*^{-/-} mouse lung lysates. Quantifications of enzyme activity are shown below the zymography. (E) Western blots and fold-changes for TGF- β 1, VEGF-A, neutrophil elastase (NE) and alpha-1-antitrypsin (A1AT) with β -actin as loading control in 2-month old FVB/NTac WT and *Ism1*^{-/-} lungs. (F) Increased expression of TGF- β 1 and VEGF-A in AMs of 2-month old FVB/NTac WT and *Ism1*^{-/-} lungs. (G) Relative reactive oxygen species (ROS) in 2-month old FVB/NTac WT and *Ism1*^{-/-} lungs. Brain tissue (positive for ISM1 expression) was used as a control to demonstrate lung specificity for ISM1 deficiency. (H) Heatmap showing altered cytokine expression in 2-month old FVB/NTac *Ism1*^{-/-} lungs. (I) Western blot and fold-changes for GM-CSF and MMP-12 with β -actin as loading control in P1, P7 and 1-month old FVB/NTac WT and *Ism1*^{-/-} lungs (including 2-months old GM-CSF and MMP-12 from Figure 2E and H). Data are mean \pm SD and were analyzed by two-group two-tailed Student's *t*-test (B–E, G and I). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. A.U.: arbitrary units. Scale bars: 20 μ m (A, F); 100 μ m (C). All figures are representatives of twice repeated experiments with similar results, *n* = 4 – 5 mice per group.

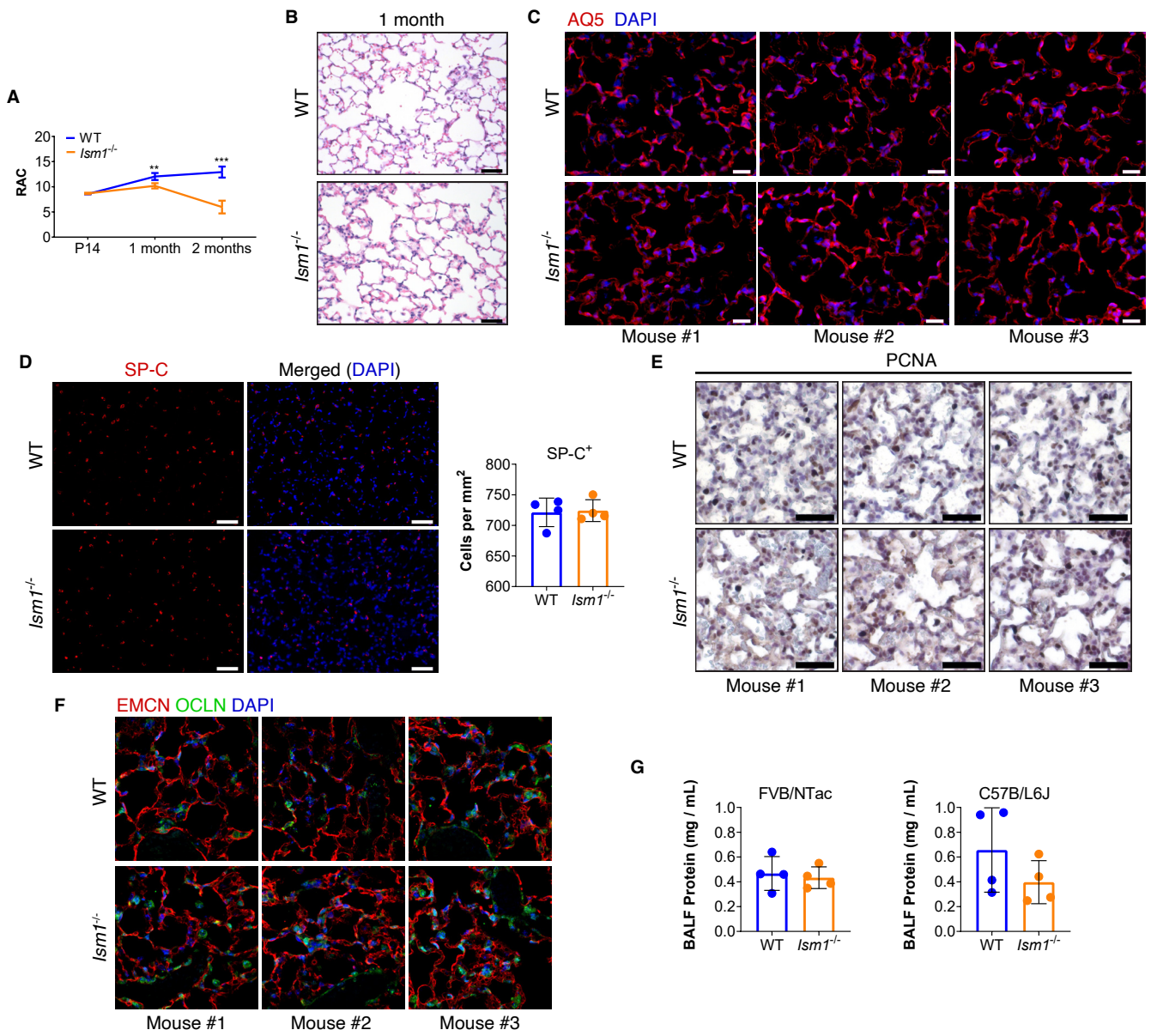


Fig. S5. Emphysema in *Ism1*^{-/-} mice is not due to impaired alveolar development. (A) Radial alveolar counts (RAC) of P14, 1-month and 2-month old FVB/NTac WT and *Ism1*^{-/-} lungs, and (B) representative 1-month old H&E stained left lungs. (C–F) Representative immunostainings for (C) aquaporin-5 (AQ5), (D) surfactant protein C (SP-C), (E) proliferating cell nuclear antigen (PCNA), (F) endomucin (EMCN) and occludin (OCLN) in 1-month old FVB/NTac WT and *Ism1*^{-/-} lungs. (G) Quantifications of BALF protein concentrations in 2-month old FVB/NTac and C57BL/6J WT and *Ism1*^{-/-} mice. Data are mean ± SD and were analyzed by two-group two-tailed Student's *t*-test (A, D and G). ***P* < 0.01, ****P* < 0.001. Scale bars: 20 μm (C), 50 μm (B, D, E). All figures are independent experiments with *n* = 3 – 4 mice per group.

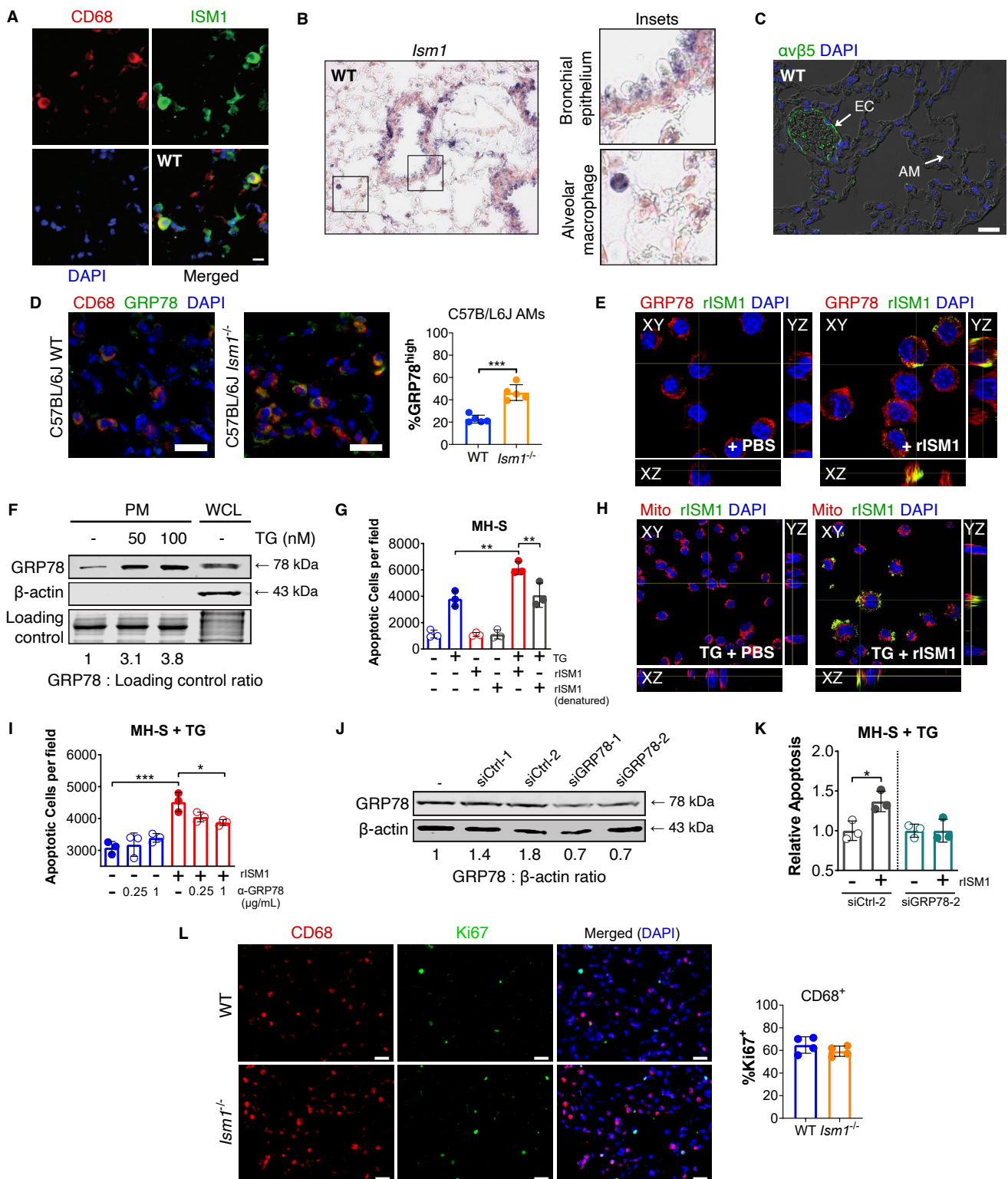


Fig. S6. ISM1 induces alveolar macrophages apoptosis through cell-surface GRP78 (csGRP78). (A) IF staining for CD68⁺ (red) cells expressing ISM1 (green) in 2-month old FVB/NTac WT lung. Nuclei is stained by DAPI (blue). (B) In-situ hybridization using anti-sense *ism1* RNA probe in 2-month WT BALB/cAnNTac mouse lung depicting ISM1 expression in the bronchial epithelium and alveolar macrophage in the insets. (C) IF staining for $\alpha 5 \beta 1$ integrin (green) and nuclei (DAPI, blue) in 2-month old FVB/NTac WT lung depicting endothelial cell (EC)-specific expression. (D) IF staining for CD68 (red), GRP78 (green) and nuclei (DAPI, blue) depicting increased AMs expressing high GRP78 in *Ism1*^{-/-} lungs. Quantifications are shown on the right as %GRP78^{high} AMs in 2-month old C57BL/6J WT and *Ism1*^{-/-} mice. (E) Confocal microscopy images showing co-localization of rISM1 (green) and cell-surface GRP78 (red) in primary AMs after 1 μ M rISM1 treatment. Nuclei stained with DAPI (blue). (F) Western blots showing upregulation of csGRP78 in MH-S mouse alveolar macrophage cells upon thapsigargin (TG) treatment. β -actin probing with coomassie blue staining indicating equal loading and high purity of the plasma membrane fractions (PM). WCL: whole-cell lysate. TG treatment is for 16 hours. (G) rISM1 induces potent apoptosis in MH-S cells after TG pretreatment. Analysis is carried out in triplicates. (H) Confocal microscopy images of TG-pretreated MH-S cells showing mitochondria (Mito: Mitotracker red) and rISM1 (green) co-localization after 1 μ M rISM1 treatment. Nuclei are stained with DAPI (blue). (Continued on next page)

(Continued from previous page) (I) Anti-GRP78 antibody neutralizes ISM-induced apoptosis of TG pretreated MH-S cells. Analysis was carried out in triplicates. (J) Western blots showing siRNA knockdown of GRP78 in MH-S cells at 24 hours post transfection. β -actin as loading control in MH-S whole-cell lysates. siCtrl-1 and siCtrl-2 are respective scrambled RNA sequences of siGRP78-1 and siGRP78-2. GRP78 : β -actin ratios are relative to untransfected MH-S cells (lane 1) (K) Apoptosis in MH-S cells after RNAi knockdown of GRP78 and 1 μ M rISM1 treatment. Analysis was carried out in triplicates. (L) IF staining demonstrate that macrophage proliferation is not significantly changed in *Ism1^{-/-}* lungs. Macrophage (CD68, red), proliferation (Ki67, green), and nuclei (DAPI, blue) are stained. Quantifications are shown on the right as %Ki67⁺ in CD68⁺ cells in 2-month old FVB/NTac WT and *Ism1^{-/-}* lungs. Six random images were taken per mouse lung. Scale bars: 10 μ m (A), 20 μ m (C, D), 50 μ m (L). Data are mean \pm SD and were analyzed by one-way ANOVA with Tukey's post hoc test (G, and I) and two-group two-tailed Student's *t*-test (D, K and L). *P < 0.05, **P < 0.01, ***P < 0.001. n = 3 – 5 mice per group. Data from E, F, H–K are representative of twice repeated experiments with similar results. Data from G is plotted using the means of thrice repeated experiments.

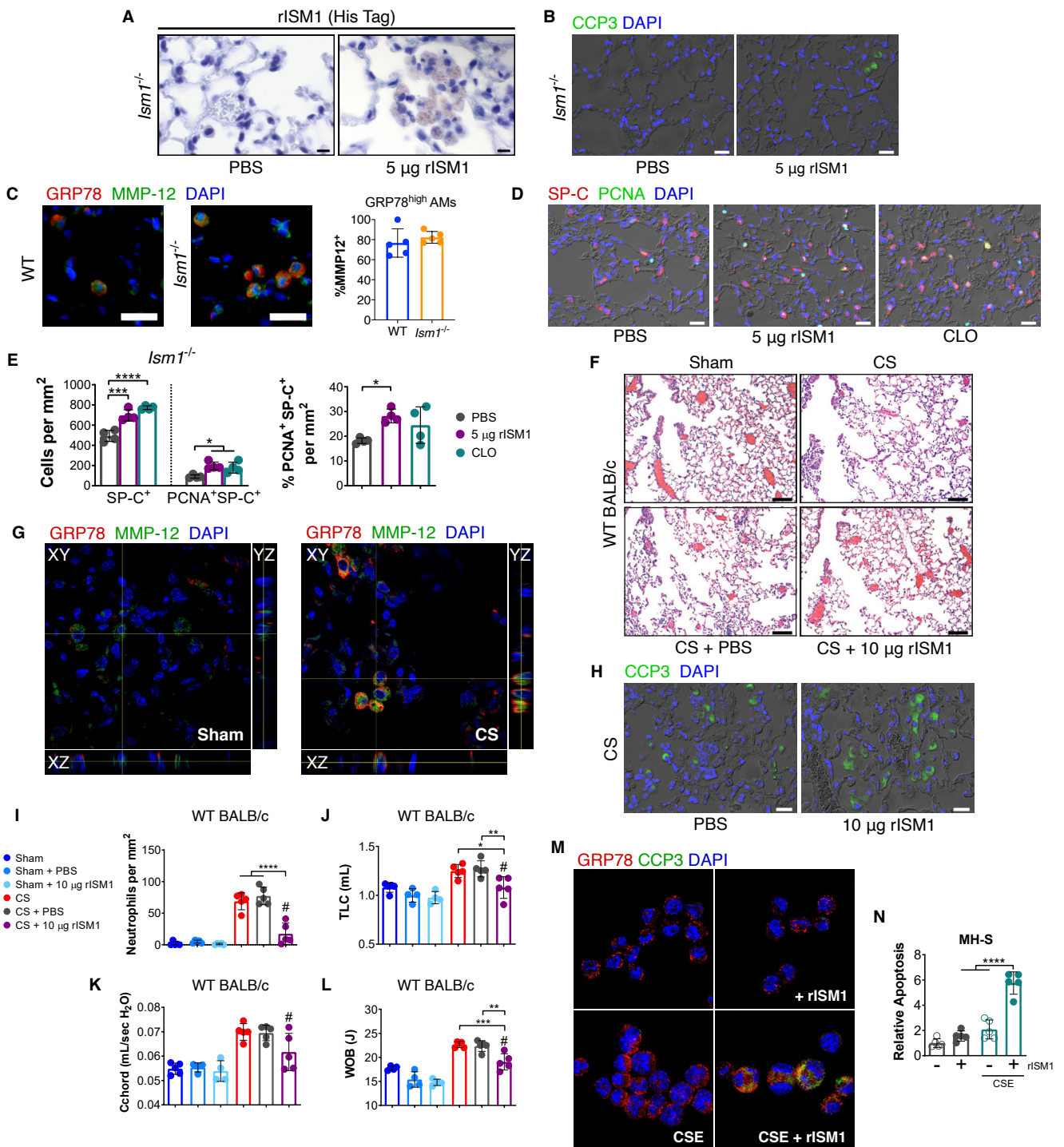


Fig. S7. rISM1 alleviates emphysema in *Ism1*^{-/-} and cigarette smoke-exposed mice. (A) Immunostainings for rISM1 and (B) cleaved caspase-3 (CCP3) in AMs of PBS and rISM1-treated FVB/NTac *Ism1*^{-/-} mice. (C) IF staining showing that high GRP78 (red) AMs are expressing MMP-12 (green) in both 2-month old FVB/NTac WT and *Ism1*^{-/-} mice. Quantifications are shown on the right as %MMP12⁺ in GRP78^{high} AMs. (D) IF staining for surfactant protein C (SP-C), PCNA, nuclei (DAPI) and (E) cell quantifications in FVB/NTac *Ism1*^{-/-} lungs after PBS, 5 μg rISM1 or liposome-clodronate (CLO) treatments. (F) H&E stained lungs of room air-exposed (Sham), cigarette smoke-exposed WT BALB/cAnNTac (WT BALB/c) mice (CS) with PBS or 10 μg rISM1 treatments. (G) Confocal microscopy images showing CS upregulated GRP78 (red) in AMs and that GRP78^{high} AMs are also MMP-12⁺ (green). Nuclei is stained by DAPI (blue). (H) IF staining for CCP3 (green) in PBS and rISM1-treated CS mouse lungs in (E). (I) Quantifications of lung neutrophil counts and (J–L) spirometry of COPD mice in (F). (J) Total lung capacity (TLC), (K) static compliance (Cchord) and (L) work of breathing (WOB). (M) IF staining for GRP78 (red), cleaved caspase-3 (CCP3, green), nuclei (DAPI, blue) and (N) quantifications for rISM1-induced apoptosis in cigarette smoke extract (CSE)-pretreated MH-S cells. Analysis was carried out using five low power microscope fields. Data are mean ± SD and were analyzed by two-group two-tailed Student's *t*-test (C) and one-way ANOVA with Tukey's post hoc test (E, I–L and N). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. #: no significant difference compared to Sham group. *n* = 4 – 5 mice per group. Scale bars: 10 μm (A), 20 μm (B, C, D, H), 100 μm (F). Data from A–E are representatives of twice repeated experiments with similar results. Data from F–N are independent experiments using the same experimental groups of mice in (F).

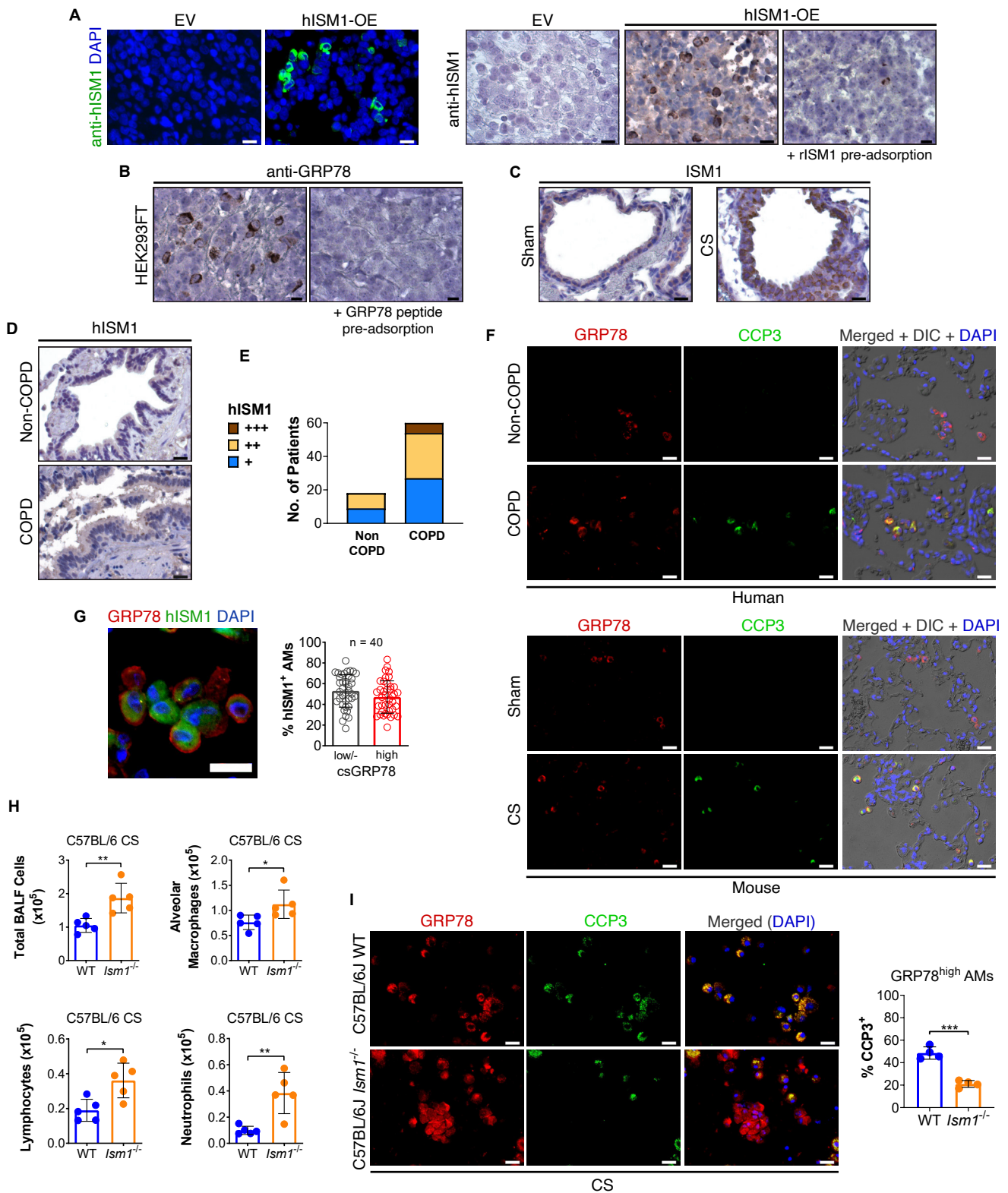


Fig. S8. ISM1 expression in COPD patients and cigarette smoke-exposed mice. (A) Anti-ISM1 antibody verification by immunostainings with anti-hISM1 antibody in empty vector (EV) or hISM1 overexpressing (hISM1-OE) HEK293FT cells with and without recombinant ISM1 (rISM1) pre-adsorption. (B) Anti-GRP78 antibody verification by immunostaining with anti-GRP78 antibody with and without GRP78 peptide pre-adsorption in normal HEK293FT cells. (C) IHC for ISM1 in the bronchial epithelium of room air-exposed (Sham) and cigarette smoke-exposed WT BALB/cAnNTac (WT BALB/c) mice (CS). $n = 5$ mice per group; and (D) non-COPD and COPD patients. (E) Distribution of hISM1 expression by COPD status. (F) IF staining for GRP78, cleaved caspase-3 (CCP3) and nuclei (DAPI) in lung tissue sections of non-COPD and COPD patients (top panel) and in room air-exposed (Sham) and cigarette smoke-exposed (CS) WT BALB/cAnNTac lungs (bottom panel). $n = 5$ mice per group. (G) IF staining for GRP78 (red) and hISM1 (green) in AMs of non-COPD and COPD patients. Quantification is shown on the right. $n = 40$ patients. (H) Quantifications of BALF cells from cigarette smoke-exposed C57BL/6J WT and *Ism1*^{-/-} mouse lungs. (I) IF staining for GRP78 (red) and CCP3 (green) in cytopsin preparations from BALF of 2-week CS-exposed C57BL/6J WT and *Ism1*^{-/-} mice, depicting reduced apoptosis of high GRP78 AMs in *Ism1*^{-/-} mice. Quantification is shown on the right. $n = 4 - 5$ mice per group. Data are mean \pm SD and were analyzed by two-group two-tailed Student's *t*-test (G, H and I). Patient sample sizes are depicted on graph. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars: 20 μm for all relevant panels, except F top panel is at 10 μm . Data from H and I are representatives of twice repeated experiments with similar results.

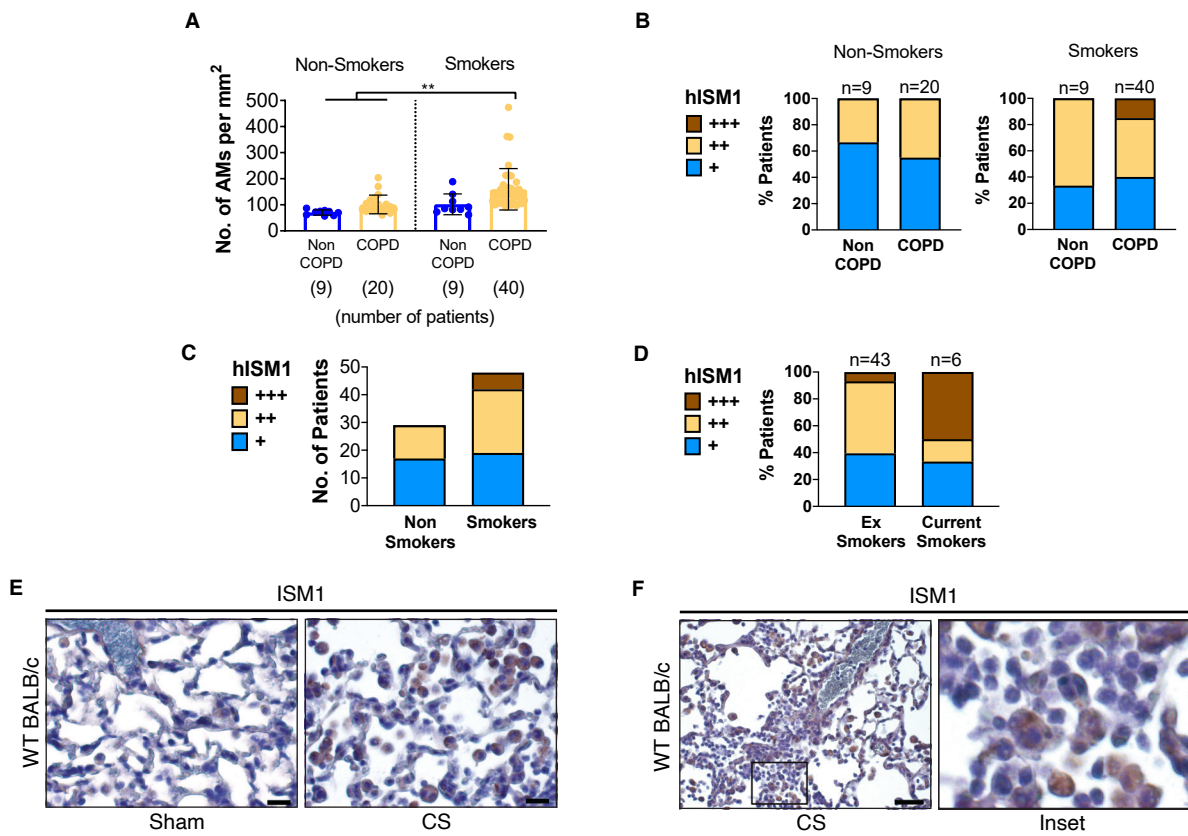


Fig. S9. ISM1 expression in AMs is associated with smoking. (A) Quantifications of AMs in non-COPD and COPD patients. (B) Distribution of hISM1 protein expression by IHC and (C) smoking status. (D) Distribution of hISM1 expression in ex and current smokers. (E) IHC staining for ISM1 expression in AMs and (F) not polymorphonuclear leukocytes or lymphocytes of room air-exposed (Sham) and cigarette smoke-exposed WT BALB/c mice (CS). $n = 5$ mice per group. Scale bars: $20 \mu\text{m}$ (E, F). Data are mean \pm SD and were analyzed by two-way ANOVA with Tukey's post hoc test (A). Patient sample sizes are depicted on graph. $**P < 0.01$.

Table S1. Patient demographics.

Demographics	COPD (n=60)	Non-COPD (n=18)
Sex (Male %)	50%	16.70%
Age	68.77 ± 0.94	54.00 ± 2.73
Current smokers (n)	6	-
Ex-smokers (n)	33	9
Non-smokers (n)	21	9
FEV₁ (% predicted)		
Current smokers	54.33 ± 8.192	-
Ex-smokers	52.26 ± 4.58	100.60 ± 4.75
Non-smokers	76.15 ± 5.45	90.89 ± 2.38
FVC (% predicted)		
Current smokers	61.33 ± 7.32	-
Ex-smokers	60.38 ± 4.25	98.44 ± 5.10
Non-smokers	79.30 ± 5.40	90.22 ± 3.04
FEV₁/FVC		
Current smokers	0.48 ± 0.05	-
Ex-smokers	0.48 ± 0.03	0.79 ± 0.02
Non-smokers	0.61 ± 0.02	0.78 ± 0.02

Forced expiratory volume in 1 sec (FEV1), Forced vital capacity (FVC). Data are mean ± s.e.m.

Table S2. Multiple linear regression analyses of factors contributing towards AM apoptosis in human lung samples.

Variables	B	SE	β	SE	P-Value (adj.)	R ² (adj.)
hISM1 expression (++)	6.140	2.437	0.273	0.108	0.014	
hISM1 expression (+++)	16.508	5.167	0.391	0.122	0.002	
GRP78 expression	0.163	0.068	0.283	0.117	0.019	
Age	-0.034	0.154	-0.032	0.142	0.825	
Sex	-2.117	2.607	-0.093	0.115	0.420	
Smoker*	-1.124	2.679	-0.048	0.115	0.676	
GOLD-1	2.190	4.780	0.079	0.172	0.648	
GOLD-2	6.793	4.460	0.268	0.176	0.132	
GOLD-3	-0.015	4.857	0.000	0.156	0.998	
GOLD-4	7.697	4.329	0.247	0.139	0.080	
						0.245

*Smokers are defined as individuals who have used at least 100 cigarettes in their lifetimes.
 B: unstandardized coefficient; β : standardized coefficient; SE: standard error; adj.: adjusted

Table S3. Chi-square test for trend between hISM1 expression and COPD status.

hISM1 expression	Non-COPD (%)	COPD (%)	Total no. of patients (%)
+	9 (25)	27 (75)	36 (100)
++	9 (25)	27 (75)	36 (100)
+++	0 (0)	6 (100)	6 (100)
Total no. of patients	18 (23.1)	60 (76.9)	78 (100)

Chi-square test for trend	
χ^2	0.798
Degrees of freedom	1
<i>P</i> -Value	0.372

Table S4. Chi-square test for trend between hISM1 expression and smoking.

hISM1 expression	Non-smoker (%)	Smoker (%)	Total no. of patients (%)
+	17 (47.2)	19 (52.8)	36 (100)
++	12 (33.3)	24 (66.7)	36 (100)
+++	0 (0)	6 (100)	6 (100)
Total no. of patients	29 (37.2)	49 (62.8)	78 (100)

Chi-square test for trend	
χ^2	4.804
Degrees of freedom	1
<i>P</i> -Value	0.028