Supplementary information for:

Resetting proteostasis with ISRIB promotes epithelial differentiation to attenuate pulmonary fibrosis

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This PDF file includes:

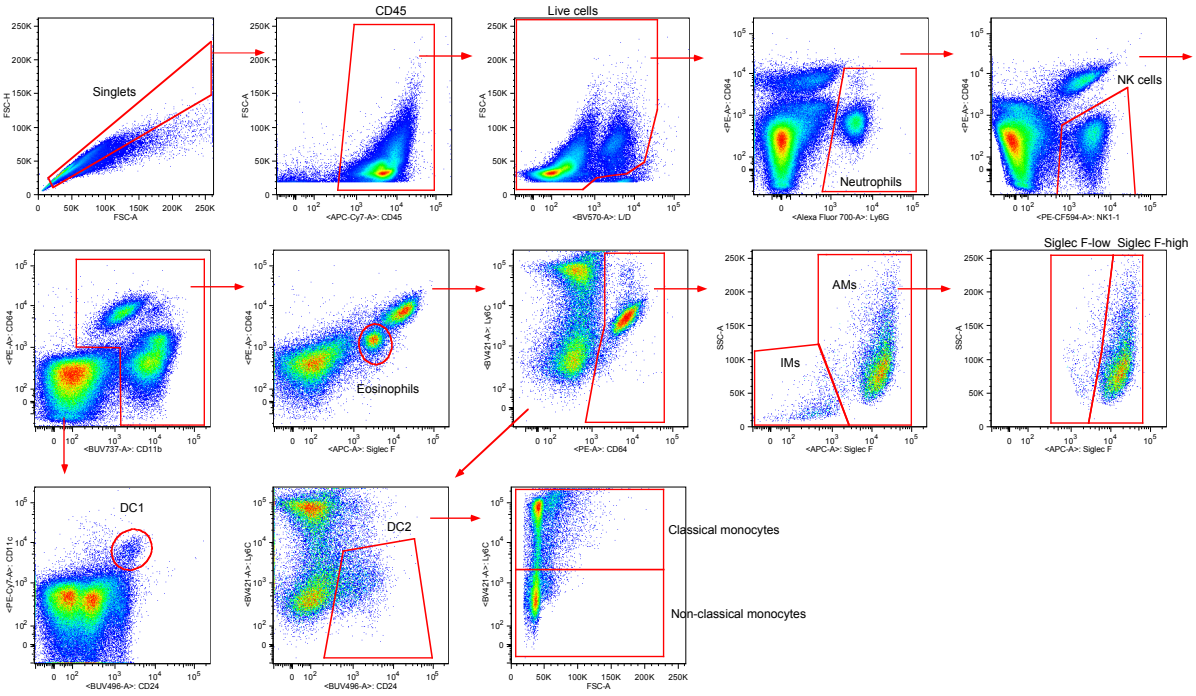
Figures S1-S7
Table S1

Other supplementary materials for this manuscript include the following:

Datasets S1–S5

SUPPLEMENTAL FIGURES AND TABLES

A Gating strategy (myeloid cells)



B Gating strategy (lymphoid cells)

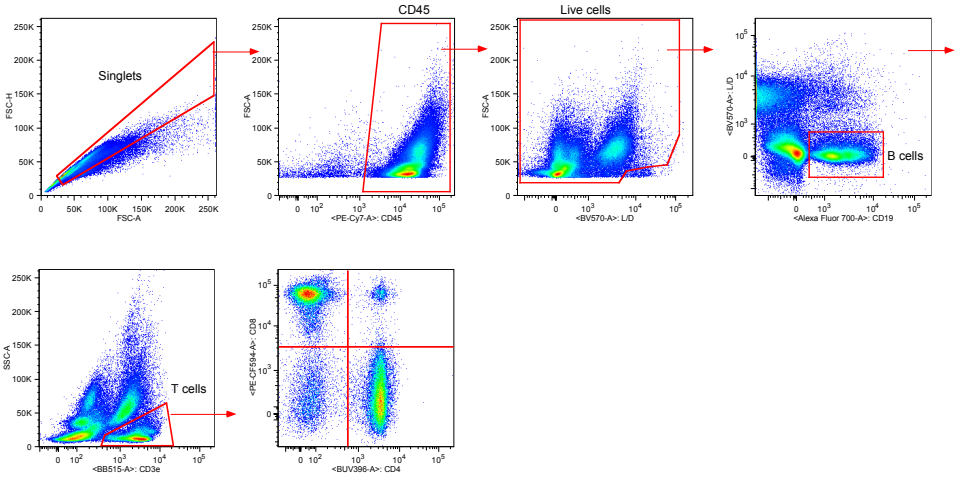


Figure S1. Gating strategy used to identify myeloid and lymphoid cell populations in single cell suspensions from the whole lung. Gating strategy for myeloid cell populations (**A**) and lymphoid cell populations (**B**). Representative flow cytometry plots from a naïve young adult mouse lung are shown.

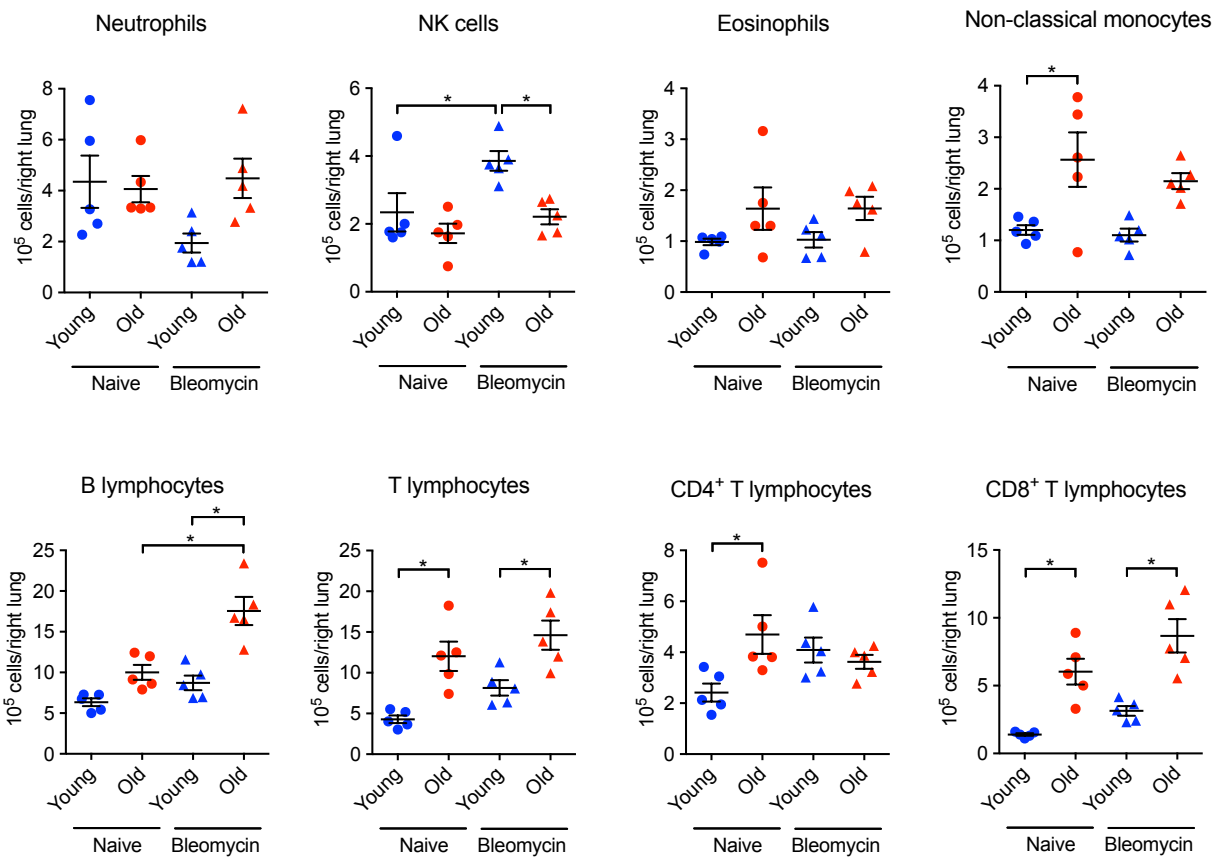


Figure S2. Lung lymphoid and myeloid cell populations in young adult and old mice. Young adult mice (3–5 months) and old mice (18–24 months) were administered bleomycin (0.025 unit/50 µl, intratracheally) and the lungs were harvested 28 days later. Myeloid populations including neutrophils, eosinophils and non-classical monocytes and lymphoid populations including B cells, $CD4^+$ T cells, $CD8^+$ T cells, and NK cells, were enumerated using flow cytometry. Data presented as mean ± SEM, 5–8 mice per group, one-way ANOVA with Tukey test for multiple comparisons. *, $p < 0.05$.

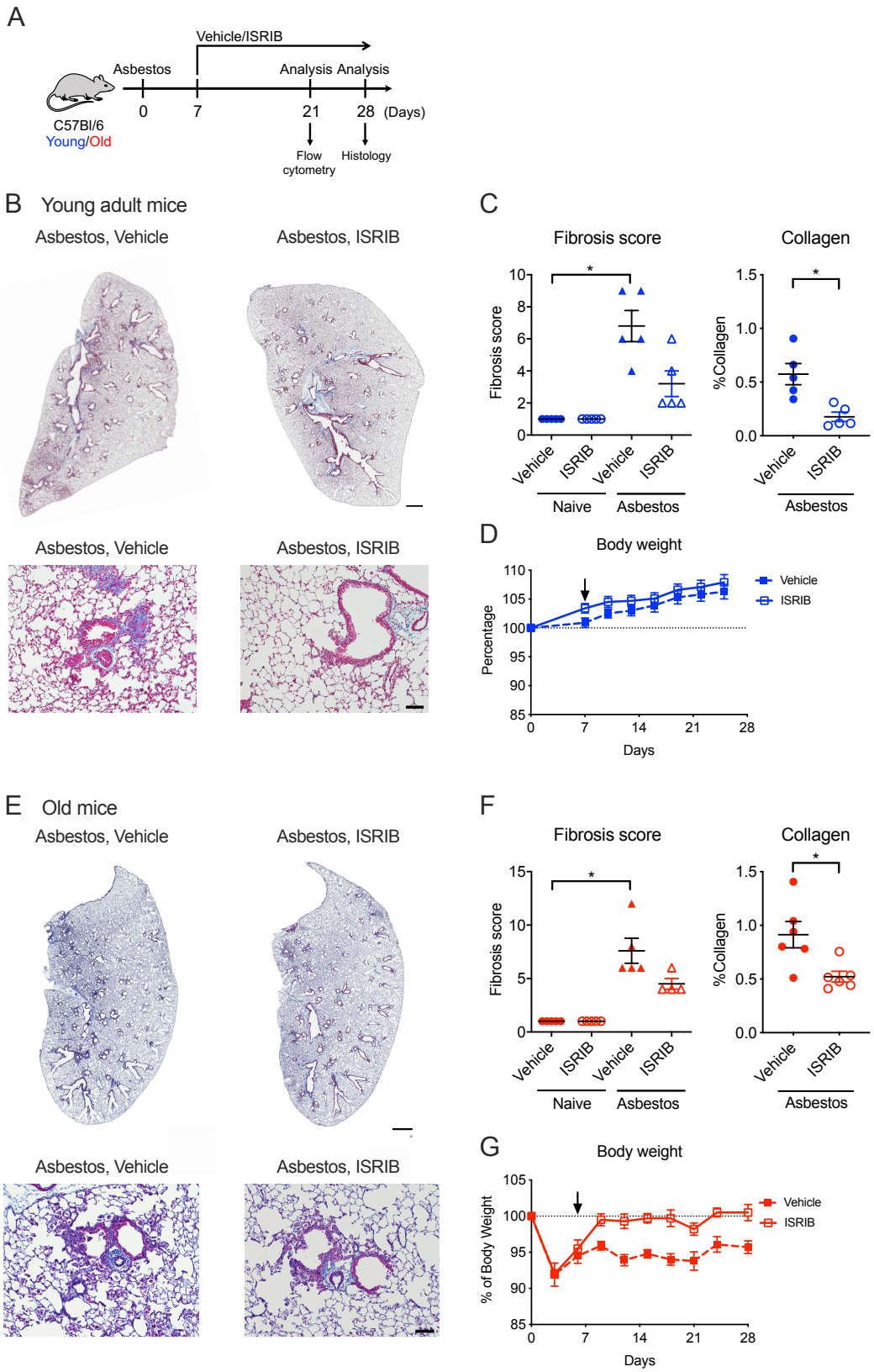


Figure S3. Therapy with ISRIB attenuates asbestos-induced lung fibrosis in young adult and old mice. (**A**) Schematic of the experimental design. Young adult (3–5 months) and old (18–24 months) mice

were administered crocidolite asbestos (100 μg/50 μl, intratracheally) and treated with 2.5 mg/kg of ISRIB or vehicle (i.p., every day) beginning at day 7 and harvested at the indicated time points. (**B, E**) Representative images of lung tissue from naïve or asbestos-treated young adult (**B**) or old (**E**) mice with or without ISRIB on day 28. Masson's trichrome staining. Scale bar = 1 mm. Representative images in high magnification are also shown. Scale bar = 100 μm. (**C, F**) Ashcroft fibrosis score (Mann-Whitney tests with Bonferroni multiple test correction) and relative collagen levels using second harmonic generation (Mann-Whitney test) from naïve mice or asbestos-treated mice with or without ISRIB. (**D, G**) Body weight. Arrow indicates start of ISRIB treatment (one-way ANOVA with Dunnet's multiple comparisons test). Data are shown as mean ± SEM, 5–7 mice per group. * $p < 0.05$. Representative data from one of two independent experiments are shown.

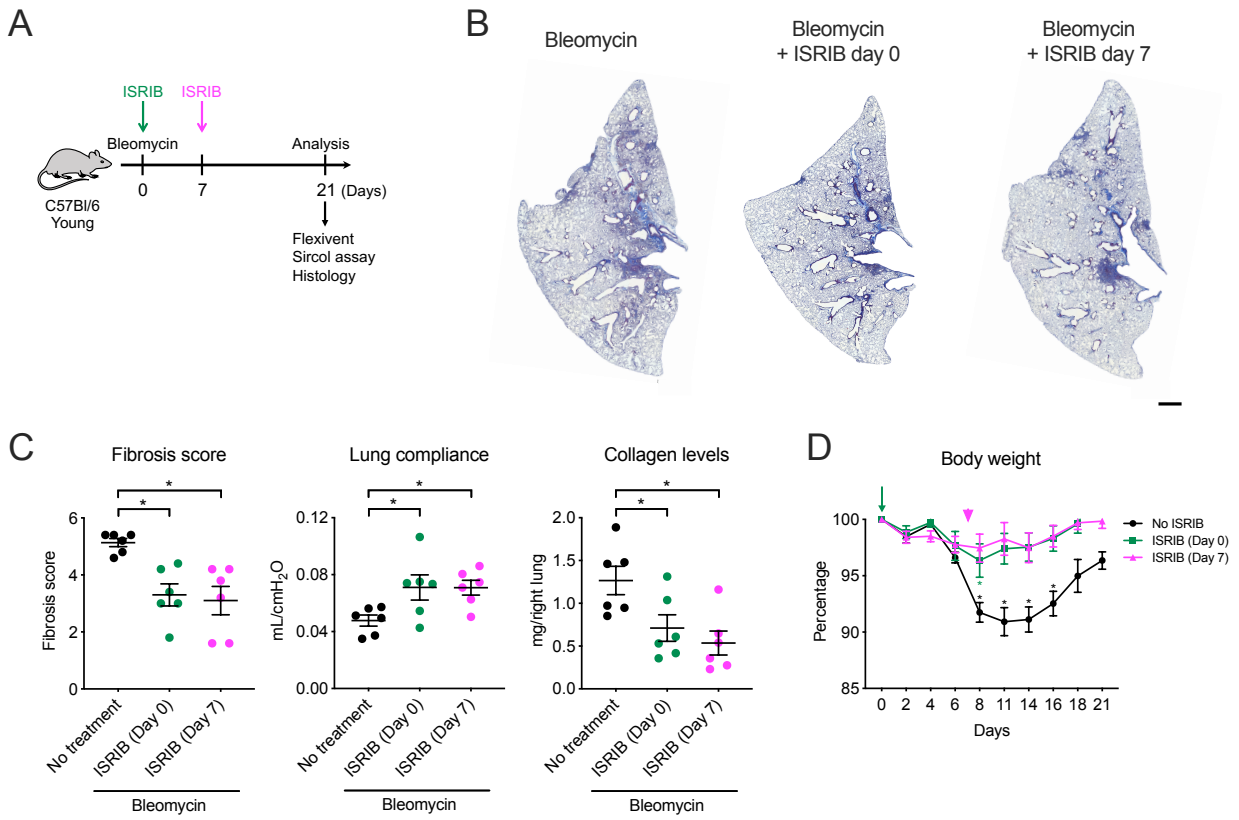


Figure S4. **A single dose of ISRIB ameliorates lung fibrosis.** (**A**) Schematic of the experimental design. Young adult (3–5 months) mice were administered 0.025 unit of bleomycin and treated with a single dose of ISRIB (2.5 mg/kg) simultaneously or 7 days later. (**B**) Representative Masson's trichrome stained lung sections (scale bar = 1 mm). (**C**) Ashcroft fibrosis score, lung compliance (Flexivent), and soluble collagen in lung homogenates. One-way ANOVA with Dunnett's test for multiple comparisons. (**D**) Body weight. Arrows indicate ISRIB treatment. one-way ANOVA with Dunnet's multiple comparisons test, comparing data from each day to baseline (day 0). Data are shown as mean ± SEM, 6 mice per group. * $p < 0.05$.

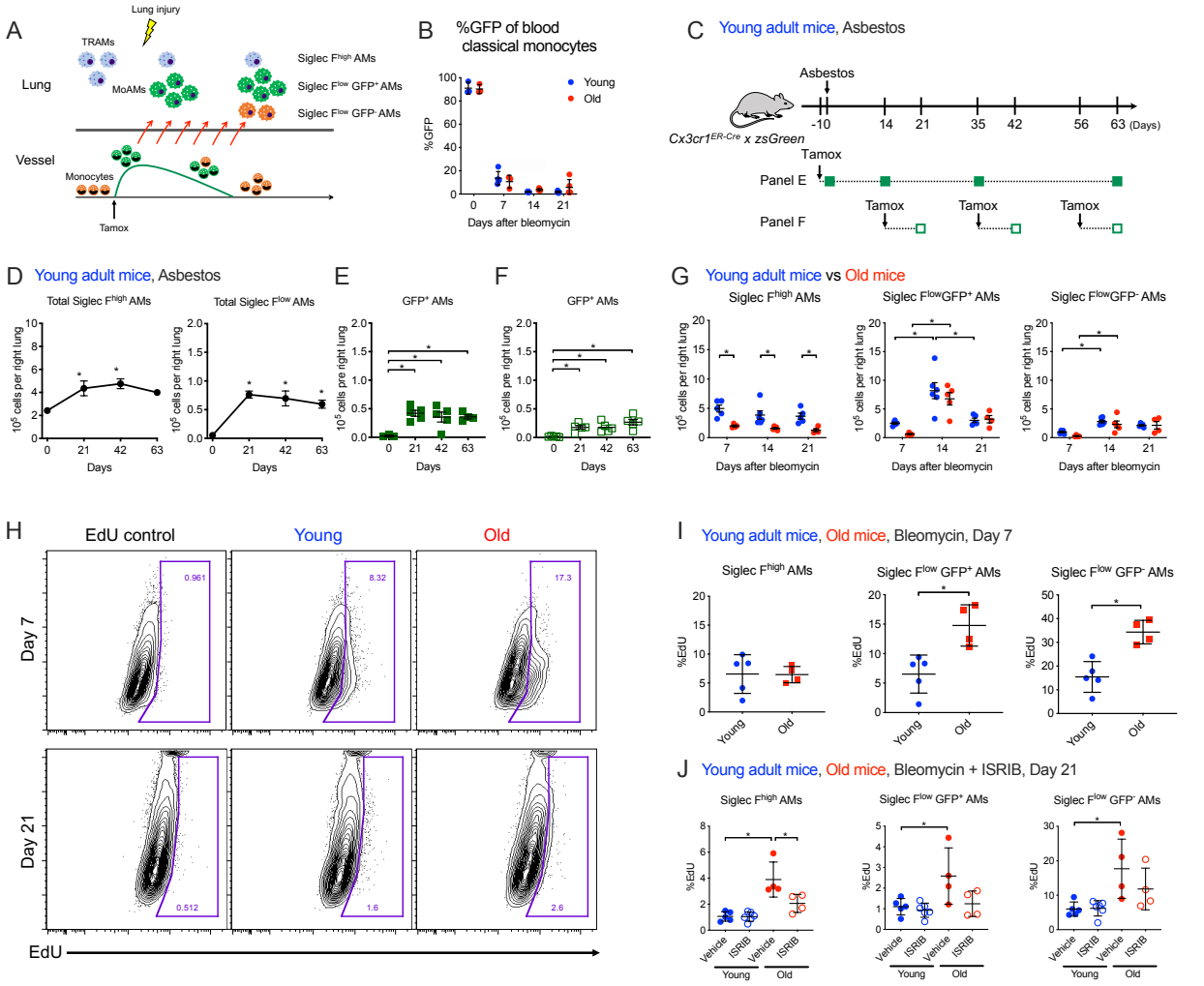


Figure S5. The recruitment kinetics of monocyte-derived alveolar macrophages in young adult and old mice during lung fibrosis. (**A**) Schematic of the *Cx3cr1*^{ERCre} × *zsGreen* lineage tracing system to assess recruitment of monocyte-derived alveolar macrophages. (**B**) Percentage of GFP-positive circulating monocytes during the course of bleomycin-induced lung fibrosis after a single dose of tamoxifen administered one day prior to bleomycin in young adult and old *Cx3cr1*^{ERCre} × *zsGreen* mice. (**C**) Schematic of the genetic lineage tracing system in the asbestos lung fibrosis model. (**D**) The number of total Siglec F^{high} alveolar macrophages (AMs), Siglec F^{low} AMs in the asbestos model. One-way ANOVA with Dunnett's test for multiple comparisons. (**E**) Monocyte-derived alveolar macrophages recruited during the first week after asbestos instillation persist during the course of asbestos-induced fibrosis (one-way ANOVA with Dunnett's test for multiple comparisons, compared to day 0). *Cx3cr1*^{ERCre} × *zsGreen* mice received a pulse of tamoxifen one day prior to instillation of asbestos and were analyzed at indicated time points. No significant difference between the number of GFP+ monocyte-derived alveolar macrophages between days 21, 42 and 63. (**F**) Recruitment of monocyte-derived alveolar macrophages continues through the course of asbestos-induced fibrosis. *Cx3cr1*^{ERCre} × *zsGreen* mice were intratracheally instilled with asbestos and were treated with a pulse of tamoxifen 7 days prior to analysis at indicated time points. No significant difference between the number of GFP+ monocyte-derived alveolar macrophages between days 21, 42 and 63, one-way ANOVA with Dunnett's test for multiple comparisons. (**G**) Young (3–5 months) and old (18–24 months) *Cx3cr1*^{ERCre} × *zsGreen* mice have similar kinetics of monocyte-derived alveolar macrophage recruitment in bleomycin-induced lung fibrosis. Tamoxifen was administered one day prior to

intratracheal instillation of bleomycin and mice were treated and harvested at the indicated time-points. Two-way ANOVA with Tukey's test for multiple comparisons. (**H**) Representative contour plots showing EdU incorporation in Siglec $F^{low}GFP^{+}$ alveolar macrophages. (**I**) EdU incorporation into monocyte-derived alveolar macrophages in old mice. Mann-Whitney test. *$p < 0.05$. (**J**) EdU incorporation into monocyte-derived alveolar macrophages with and without ISRIB treatment. Kruskal-Wallis test with Dunn's correction for multiple comparisons. *$p < 0.05$. Data are shown as mean ± SEM.

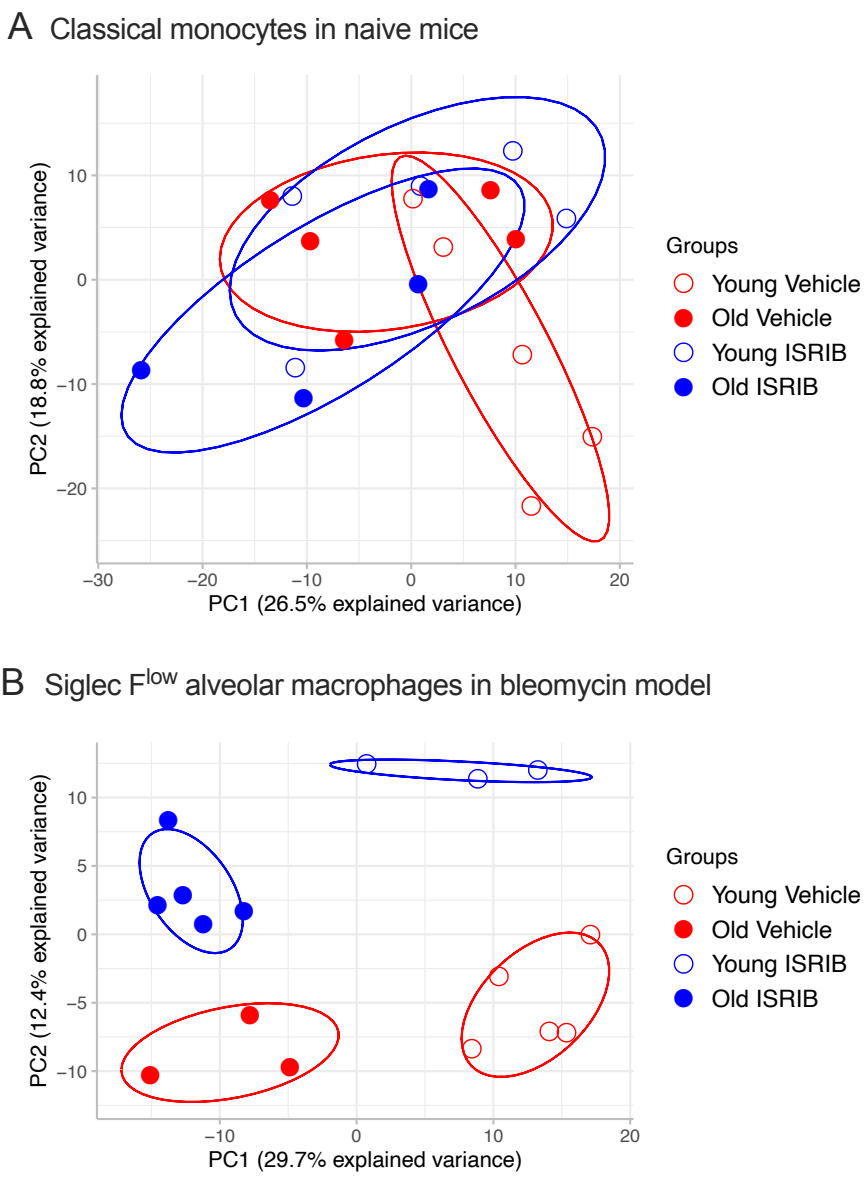


Figure S6. Transcriptomic profiling does not reveal evidence of activation of the ISR in monocyte or alveolar macrophage populations in the lung. (**A**) Principal component analysis of classical monocytes in young and old naïve mice treated with ISRIB or vehicle for 7 days did not reveal age- or treatment-related sources of variation. (**B**) Principal component analysis of CD64^{+}Siglec F^{low} monocyte-derived alveolar macrophages in young adult and old mice exposed to bleomycin with and without ISRIB treatment.

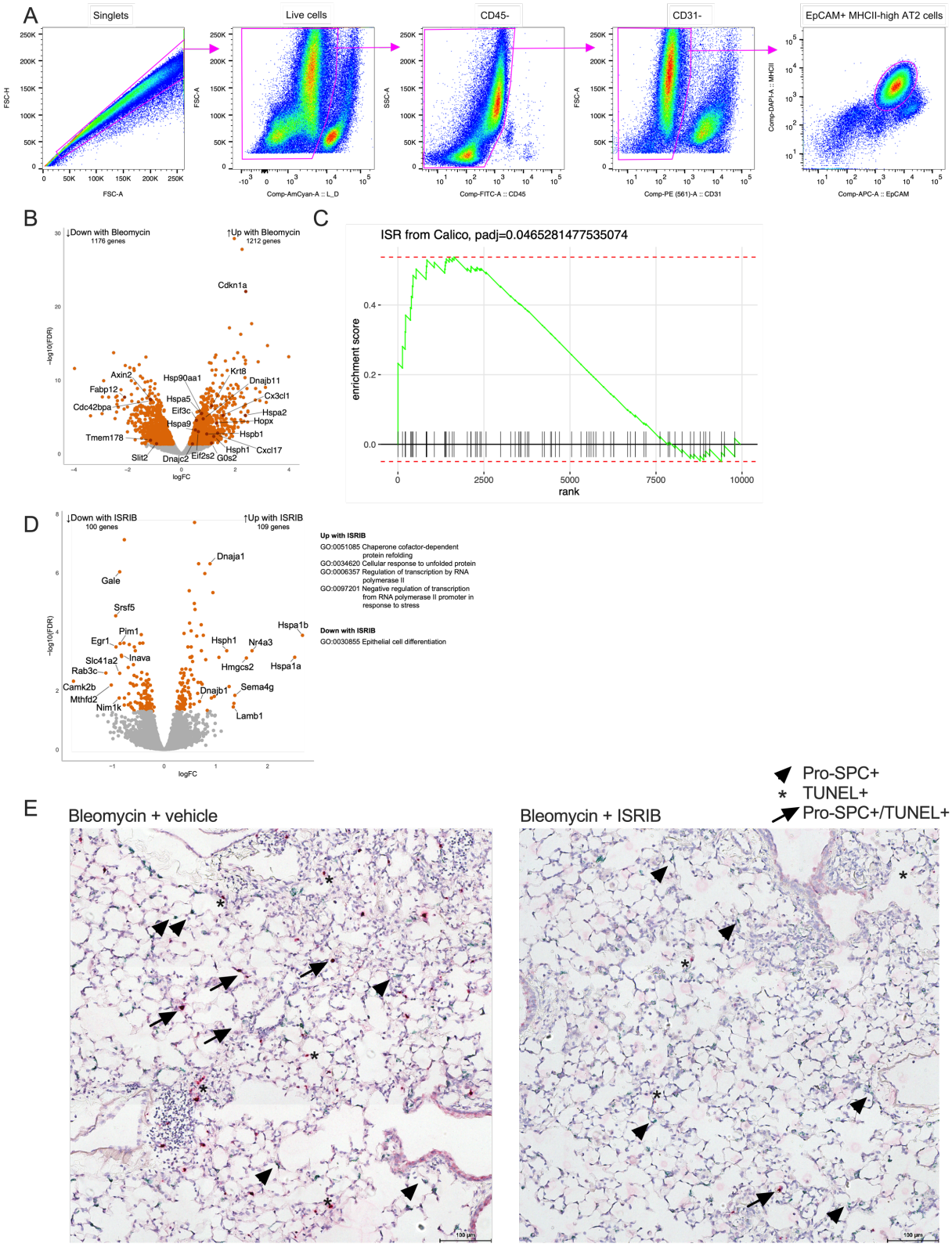


Figure S7. ISRIB ameliorates fibrosis by modulating stress response in epithelial cells. (**A**) Representative gating strategy to isolate AT2 cells by flow sorting. (**B**) Volcano plot showing up- and down-regulated genes in AT2 cells 14 days after treatment with bleomycin (FDR q-value < 0.05). (**C**) GSEA

enrichment plot of genes related to integrated stress response in AT2 cells after treatment with bleomycin (44). (**D**) Volcano plot showing up- and down-regulated genes in AT2 cells in young naïve mice 24 hours after a single dose of ISRIB. (**E**) Representative microphotographs of lung tissue stained for TUNEL and prosurfactant protein C (Pro-SPC). Mice received intratracheal administration of bleomycin at day 0, were treated with vehicle or ISRIB on day 7 and analyzed on day 10. Arrowheads indicate Pro-SPC+ AT2 cells. Asterisks indicate TUNEL+ apoptotic cells. Arrows indicate $TUNEL^{+}Pro\text{-}SPC^{+}$ apoptotic AT2 cells. Scale bar = 100μm.

Supplemental Table S1: Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat anti-mouse I-A/I-E (MHC II), BUV395	BD Biosciences	Cat# 743876, RRID:AB_2741827
Rat anti-mouse CD24, BUV496	BD Biosciences	Cat#564664, RRID:AB_2716853
Rat anti-mouse CD11b, BUV737	BD Biosciences	Cat#564443, RRID:AB_2738811
Rat anti-mouse Ly-6C, eFluor 450	eBioscience	Cat#48-5932-82, RRID:AB_10805519
Mouse anti-mouse CD64, PE	BioLegend	Cat#139303, RRID:AB_10613467
Mouse anti-mouse NK-1.1, PE-CF594	BD Biosciences	Cat#562864, RRID:AB_2737850
Hamster anti-mouse CD11c, PE-Cy7	BD Biosciences	Cat#558079, RRID:AB_647251
Rat anti-mouse Siglec F, Alexa Fluor 647	BD Biosciences	Cat#562680, RRID:AB_2687570
Rat anti-mouse CD45, APC-Cy7	BD Biosciences	Cat#557659, RRID:AB_396774
Rat anti-mouse CD45, FITC	BD Biosciences	Cat#561088, RRID:AB_10562038
Rat anti-mouse CD45, eFluor 450	eBioscience	Cat#48-0451-82, RRID:AB_1518806
Rat anti-mouse Ly-6G, Alexa Fluor 700	BD Biosciences	Cat#561236, RRID:AB_10611860
Armenian hamster anti-mouse CD3e, FITC	eBioscience	Cat#11-0031-82, RRID:AB_464882
Rat anti-mouse CD25, PE	eBioscience	Cat#12-0251-82, RRID:AB_465607
Rat anti-mouse CD8a, PE	BioLegend	Cat#100762, RRID:AB_2564027
Rat anti-mouse CD62L, APC-eFluor 780	eBioscience	Cat#47-0621-82, RRID:AB_1603256
Rat anti-mouse CD19, Alexa Fluor 700	eBioscience	Cat#56-0193-82, RRID:AB_837083
Rat anti-mouse CD4, BUV395	BD Biosciences	Cat#563790, RRID:AB_2738426
Rat anti-mouse CD44, BUV737	BD Biosciences	Cat#564392, RRID:AB_2738785
Rat anti-mouse CD31, PE	BioLegend	Cat#102407, RRID:AB_312902
Hamster anti-mouse gp38 (T1a), PE	BioLegend	Cat#127407, RRID:AB_2161929
Hamster anti-mouse gp38 (T1a), PE-Cy7	BioLegend	Cat#127411, RRID:AB_10613294
Rat anti-mouse EpCAM, PE-Cy7	BioLegend	Cat#118216, RRID:AB_1236477
Rat anti-mouse EpCAM, APC	eBioscience	Cat#17-5791-82, RRID:AB_2716944
Rat anti-mouse Cytokeratin 8 (Krt8)	DSHB	Cat# TROMA-I, RRID:AB_531826
Rabbit anti-mouse Pro-SPC	Millipore	Cat#AB3786, RRID:AB_91588
Goat anti-mouse Podoplanin	R&D systems	Cat#AF3244, RRID:AB_2268062
Donkey anti-rabbit Alexa Flouor 488	Thermo Fisher Scientific	Cat#A21206, RRID:AB_2535792
Donkey anti-goat IgG AF568	Thermo Fisher Scientific	Cat#A11057, RRID:AB_2534104
Donkey anti-Rat Alexa Fluor 647	Abcam	Cat#ab150155, RRID:AB_2813835
Chemicals, Peptides, and Recombinant Proteins		
Bleomycin	Fresenius Kabi	Cat#0703-3154-01
Asbestos (UICC crocidolite)	Provided by US Environmental Protection Agency	
ISRIB	AdooQ BioScience	Cat#A14302
Critical Commercial Assays		
EdU	ThermoFisher Scientific	Cat#C10044
Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit	ThermoFisher Scientific	Cat#C10634
PicoPure RNA Isolation Kit	ThermoFisher Scientific	Cat#KIT0204
AllPrep DNA/RNA Mini Kit	Qiagen	Cat#80204
NEBNext RNA Ultra I kit	New England Biolabs	Cat#E7530L
polyA mRNA isolation module	New England Biolabs	Cat#E7490S

SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian kit	Takara Bio	Cat#634413
Deposited Data		
Bulk RNA-seq	This study	GSE145295, GSE145590, GSE145771
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J (8-12 weeks old)	The Jackson Laboratory	JAX: 000664
Mouse: C57BL/6J (over 18 months old)	National Institute of Aging	
Mouse: Cx3cr1ERCre	The Jackson Laboratory	JAX: 020940
Mouse: SftpcERCre	The Jackson Laboratory	JAX: 028054
Mouse: ZsGreen	The Jackson Laboratory	JAX: 007906
Software and Algorithms		
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/
bcl2fastq 2.19.1		https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html
FastQC		https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Trimmomatic 0.36		http://www.usadellab.org/cms/?page=trimmomatic
STAR 2.6.0	Dobin et al., 2013	https://github.com/alexdobin/STAR
htseq-count 0.11.2	Anders et al., 2014	https://htseq.readthedocs.io/en/latest/
edgeR 3.28.0	Robinson, 2010	

LIST OF SUPPLEMENTAL DATASETS

Dataset S1: Number of differentially-expressed genes between young/old ISRIB/vehicle samples of sorted monocytes. Related to Figure S6A.

Dataset S2: List of differentially-expressed genes, their clusters and statistics from Figure 6A.

Dataset S3: List of GO biological processes for each cluster in Figure 6A.

Dataset S4: List of differentially-expressed genes and their statistics from Figure 7B.

Dataset S5: List of differentially-expressed genes and their statistics from Figure 7D.