SUPPLEMENTARY DATA

CSF1R inhibition prevents radiation pulmonary fibrosis by depletion of interstitial macrophages

Lydia MEZIANI[1-2], Michele MONDINI[1-2], Benoît PETIT[3], Alexandre BOISSONNAS[4,5], Vincent THOMAS de MONTPREVILLE [2-7], Olaf MERCIER[2-8], Marie-Catherine VOZENIN[*3] and Eric DEUTSCH[*1-2-6]

* These authors contributed equally to this work

SUPPLEMENTARY MATERIALS AND METHODS

Human tissue samples

Four patients (1 women and 3 men) had preoperative radiotherapy and received between 35 and 60Gy before left pneumonectomy extended to the spine for Ewing sarcoma (n=1), right superior sulcus tumor (n=1), right upper lobe sleeve resection (n=1) for lung cancers, and extrapleural pneumonectomy extended to the chest wall for an epithelioid mesothelioma (n=1). Six patients (1 woman and 5 men) without radiotherapy were also included, they underwent surgery for right upper lobectomy (n=3), right lower lobectomy (n=1), middle lobectomy (n=1), left upper lobectomy (n=1) for lung cancers, metastasis or neuroendocrine tumor and were used for control purposes.

Animals and IR procedure

The mice were sampled at 3, 6, and 15 days post-IR to examine the acute response and 20 weeks post-IR to assess the late response (**supplementary Fig S7**). Aged-matched sham-irradiated (0 Gy) animals were included and compared with irradiated groups at each time point (3–6 mice per time point). Animal procedures were performed according to the protocols approved by the Ethical Committee CEEA 26 and in accordance with recommendations for the proper use and care of laboratory animals.

Cone beam computed tomography (CBCT) imaging

Irradiated (n=4) at 16Gy and non-irradiated (n=5) C57BL/6J mice underwent CBCT scan at lung level at several time points after IR. During scanning, mice were immobilized through anesthesia (2% isoflurane). The tube voltage was 40kV, tube current was 13mA (X-Rad SmArt machine, PXI). The scan was operated on 3 windows, that displayed axial, sagittal and coronal slices. ClearCanvas was used to quantify lung density on coronal slice using Hounsfield Unit (HU).

Histopathological analyses and immunohistochemistry

The mouse organs were fixed in 4% PFA, paraffin embedded and cut into 4-µm sections. The sections were stained with hematoxylin-eosin-saffran (HES) or Sirius Red (SR) and scanned using a microscopy virtual slide system (Olympus VS120).

The quantification of Lung fibrosis was assessed by the automated histological image analysis developed by Biocellvia (Marseille, France). This method, based on the evaluation of pulmonary tissue density by means of a dedicated software program, allows an operator-independent unbiased identification and quantification of fibrotic lesions in lung parenchyma (Gilhodes *et al*, 2017). Briefly, histological image analysis was performed from digital image of entire HES lung sections, excluding the walls of large bronchi located in the vicinity of the lung lobes, small bronchi and vessels (diameter >200 µm) associated with alveolar parenchyma as well as their surrounding collagen fibers. The high tissue density frequency (HDF) index was used to quantify fibrotic alterations in alveolar parenchyma. HDF index determined from the high tissue density values located specifically in fibrotic lesions allows to access to an accurate quantification of the severity of lung fibrosis (Gilhodes et al, 2017). Collagen expression was assessed by quantification of Sirius Red staining using ImageJ software.

For detection of human macrophages, paraffin human lung sections were processed for heat-induced antigen retrieval, incubated with mouse monoclonal anti-human CD163 antibody (*Diagnostic BioSystems, USA,* 1:100). Staining was visualized using the peroxidase/diaminobenzidine Mouse PowerVision kit (*ImmunoVision Technologies*) and examined using microscopy virtual slide system (Olympus VS120)

Protein isolation and Western blotting

Half of the right lung and Fibroblats co-cultured with *in vitro* activated pulmonary macrophages were lysed in RIPA buffer containing protease and phosphatase inhibitors (Roche) for Western blotting. At the indicated time point, Western blotting was performed

after electrophoresis using 10% Tris-HCl SDS-PAGE, and electrotransferred to nitrocellulose membranes (Biorad). The membranes were blocked with TBS-Tween containing 0,1%-5% BSA (Sigma) and incubated with primary antibodies, including anti-PAI-1 (1:1000; Cell Signaling); anti-Smad2/3 (1:100; Santa Cruz); anti-PhosphoSmad2/3 (1:1000;Cell Signaling) and αSMA (1:200; Abcam). The membranes were incubated with the corresponding HRP-conjugated secondary antibody (GE Healthcare Life Sciences; diluted at 1:5000 in TBST containing 5% BSA). The reactive proteins were visualized using chemiluminescence detection. The images were acquired using GeneSys imagers coupled to Synoptic 1.4MP cameras (Ozyme). The membranes were incubated with either rabbit monoclonal GAPDH (1:1000; Millipore) or mouse monoclonal Vinculin (1:4000; Abcam) to normalize the chemiluminescence levels and exposure times.

The reactive proteins were visualized using chemiluminescence detection. The images were acquired and quantified using GeneSys imagers coupled to Synoptic 1.4MP cameras (Ozyme).

Bronchoalveolar lavage (BAL)

After cervical dislocation, the tracheas were cannulated and secured using a silk suture. Phosphate-buffered saline (PBS) (500 µl) was slowly delivered and retrieved through the cannula, the lavage was repeated five times. BAL cells were treated with red blood cell lysis buffer (Gibco), washed in PBS and stored on ice until subsequent analysis.

Lung dissociation

Lungs were digested using the Lung Dissociation Kit (Miltenyi Biotec). After 30 minutes of rocking at 37°C, the lung solution was passed through a nylon mesh (70 µM; BD Falcon). The cells were treated with red blood cell lysis buffer (Gibco), washed in PBS and stored on ice until subsequent analysis.

Cytokine profiling

For the simultaneous determination of the relative levels of selected mouse cytokines and chemokines, the Mouse Cytokine Array Panel A kit (R&D Systems) was used. The assay was performed according to the manufacturer's instructions. The reactive proteins were visualized using chemiluminescence detection. The images were acquired and quantified using GeneSys imagers coupled to Synoptic 1.4MP cameras (Ozyme).

Giemsa staining

IMs and AMs from irradiated and non-irradiated lung were sorted by FACS, 15 weeks post-IR and stained with Giemsa Stain, followed by analysis using conventional light microscopy.

Fibroblasts/Macrophages co-culture

Sorted IMs and AMs from RIF were co-cultured (10^4/insert) for 24 hours with fibroblasts (5.10^3 cells/well) in a transwell system containing complete culture medium (DMEM-F12) containing 1% (v/v) antibiotics and 2% (v/v) fetal bovine serum (FBS).

Sorted IMs and AMs from healthy lungs were activated using either recombinant IFNγ (100ng/mL) or recombinant IL13/IL4 (100ng/mL for each cytokine) for 24 hours and then co-cultured (10^5/insert) for 48 hours with fibroblast (5.10^3 cells/well) in a transwell system containing complete culture medium (DMEM-F12) containing 1% (v/v) antibiotics and 2% (v/v) fetal bovine serum (FBS).

Immunofluorescence

Serial slides (4 µm) were examined in situ for fibrogenic factor expression using an anti-TGF-β1 rabbit polyclonal antibody (diluted at 1:50 in PBS containing 5% BSA, Santa Cruz Biotechnology, Inc.). The co-expression of TGF-β1 and αSMA was also examined in fixed fibroblasts using an anti-TGF-β1 rabbit polyclonal antibody (diluted at 1:100 in PBS containing 5% BSA, Santa Cruz Biotechnology, Inc.) and αSMA mouse monoclonal antibody (diluted 1/200 in PBS containing 5% BSA, Abcam). The corresponding secondary conjugated antibodies (anti-rabbit Alexa Fluor 568 and anti-mouse Alexa Fluor 488, Invitrogen) were

diluted at 1:2000 in PBS containing 5% BSA. After staining, the tissue sections and fibroblasts were examined using a confocal microscope equipped with a JVC color video camera coupled to an imaging analysis system (Leica).

Sircol assay for collagen detection

For the detection of the relative production of ECM in fibroblast supernatant, the SIRCOL Collagen assay kit (Tebu-bio) was used. The assay was performed according to the manufacturer's instructions. Absorbance was measured using Bio-Rad microplate reader 680. Collagen concentrations were obtained from standard curve.

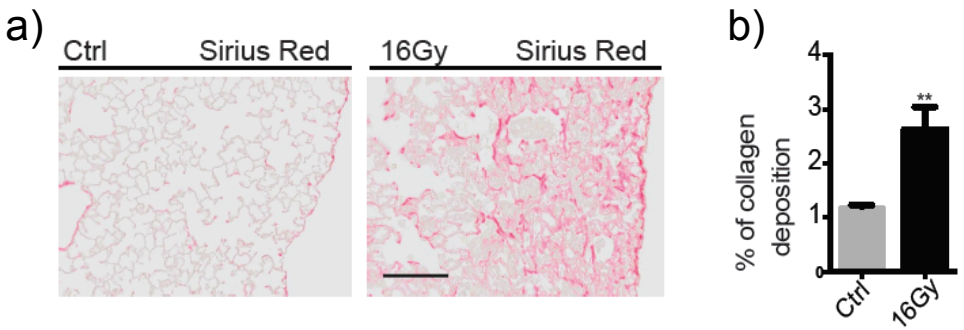
Fibroblasts isolation/culture

Whole lungs were removed from non-irradiated and irradiated mice 15 days post-IR and digested using the Lung Dissociation Kit (Miltenyi Biotec). After 30 minutes of rocking at 37°C, the lung solution was transferred to DMEM-F12 medium containing 1% (v/v) antibiotics and 20% (v/v) fetal bovine serum (FBS). After a minimum of two passages, the homogenous fibroblast populations were transferred to 12-well culture plates for subsequent experiments.

SUPPLEMENTARY FIGURES AND LEGENDS

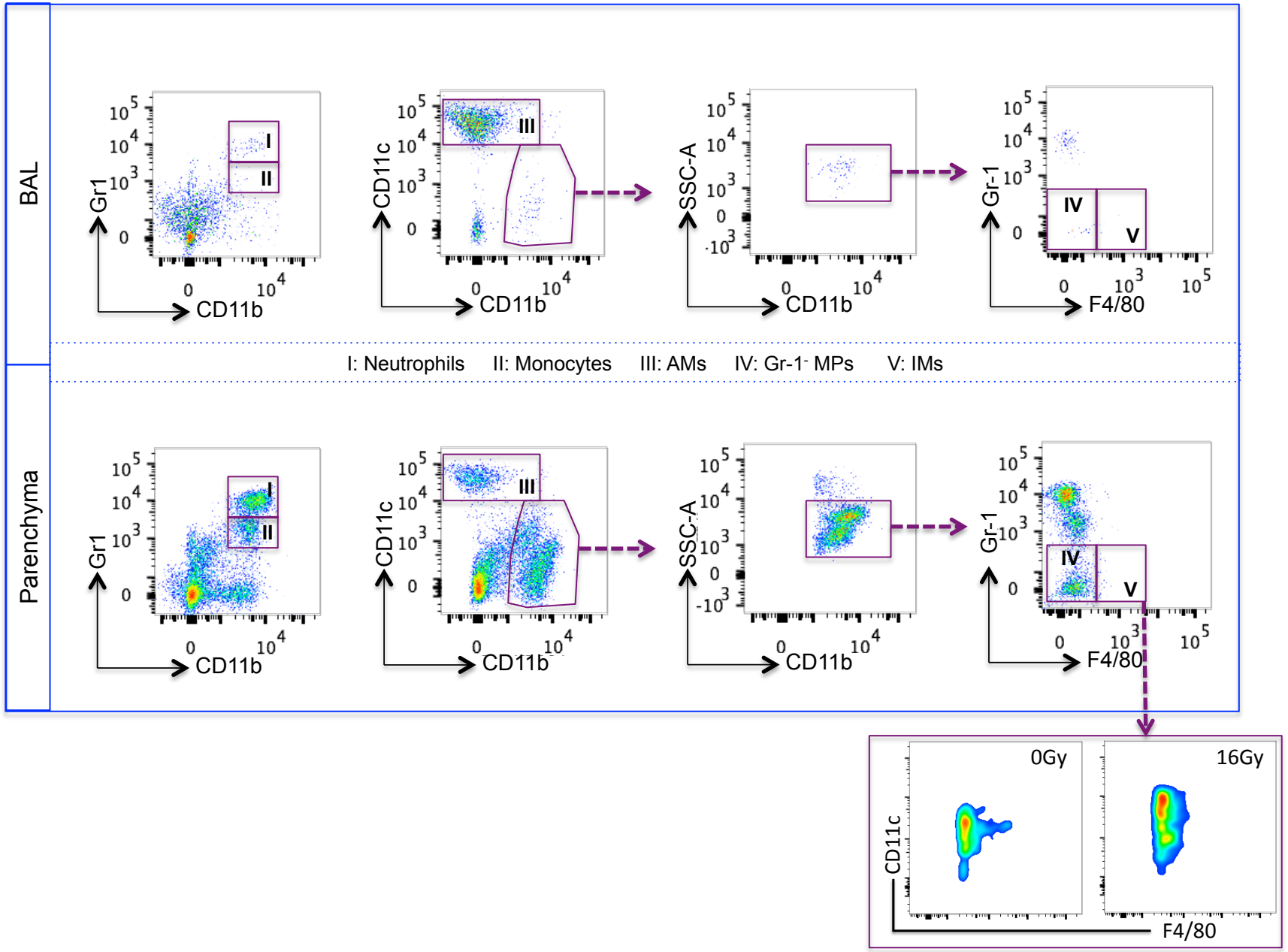
Supplementary figure S1. Fibrosis score assessment by Sirius Red staining. a) Sirius red staining of lung section from non-irradiated and irradiated (16Gy) C57BL/6 mice at 20 weeks post-IR. Scale bar: 100 μm. b) Lung fibrosis score quantification, presented as the percent of collagen deposition. Data are from one experiment presented as mean±SEM. **P<0.005 (Student's *t*-test).

Supplementary figure S1. Fibrosis score assessment by Sirius Red staining



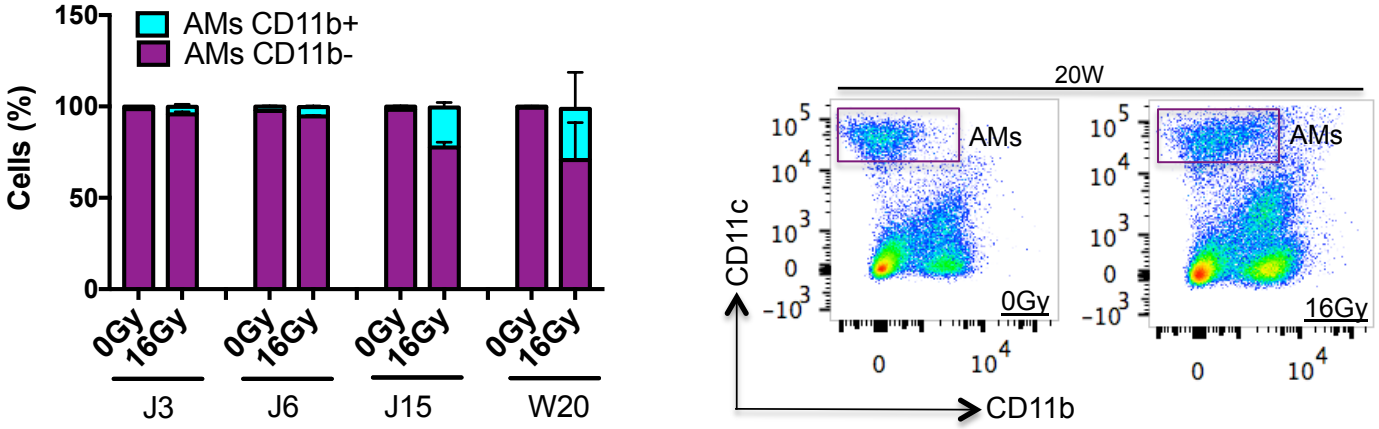
Supplementary Figure S2. Gating strategy to characterize pulmonary inflammatory cell subpopulations. Characterization of neutrophils ($Gr1^{+}$ $CD11b^{+}$), monocytes ($Gr1^{intr}$ $CD11b^{+}$) AMs ($CD11c^{+}$ $CD11b^{-}$), $Gr1^{-}$ MPs ($Gr1^{-}$ $F4/80^{-}$) and IMs ($Gr1^{-}$ $F4/80^{+}$) in BAL and lung parenchyma of C57BL/6 mice using flow cytometry.

Supplementary figure S2. Gating strategy to characterize pulmonary inflammatory cell subpopulations



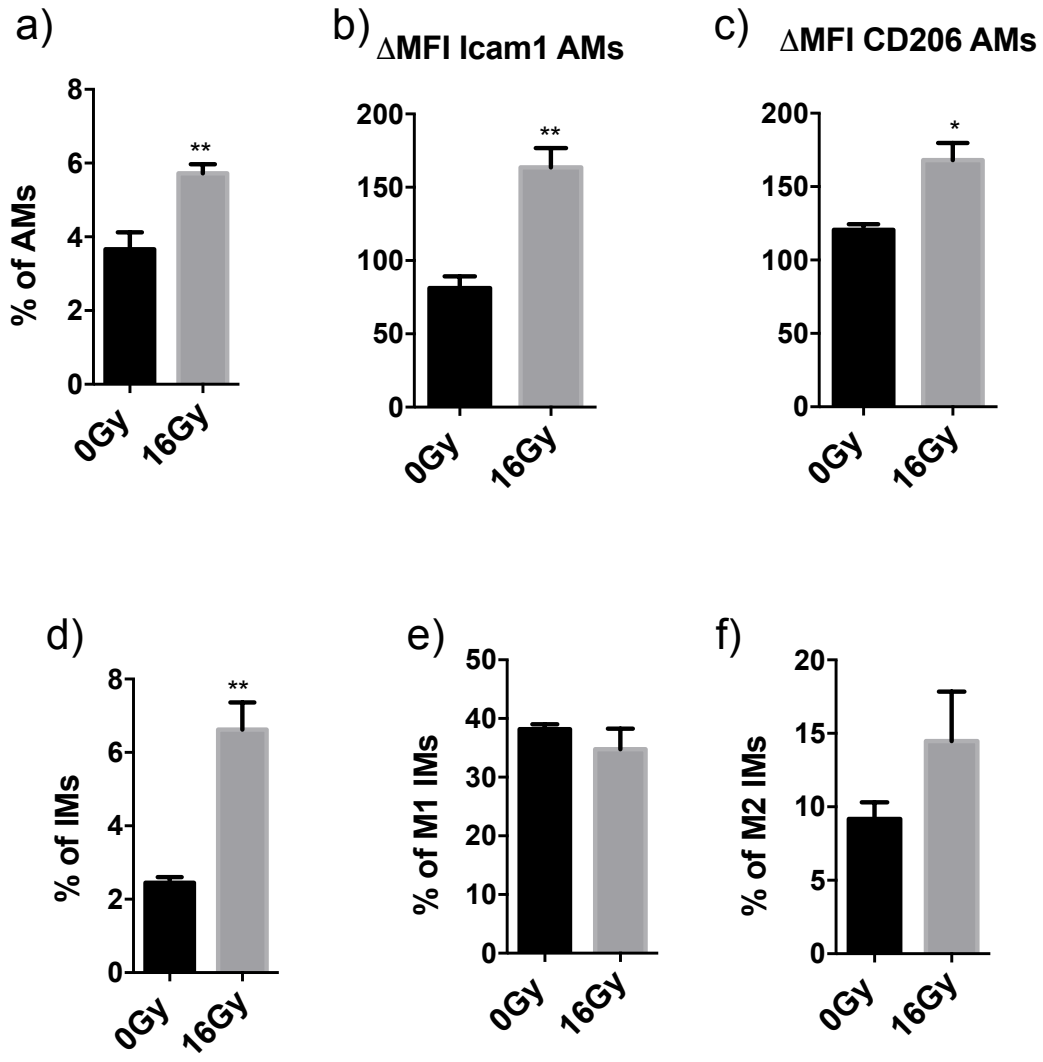
Supplementary Figure S3. AMs CD11b^{-} *versus* AMs CD11b^{+}. Percent of AMs expressing or no CD11b marker at different time points after irradiation (16Gy). AMs gating from non-irradied and irradiated lung at week 20 after irradiation. Data were obtained from two independent experiments presented as mean±SEM. Day 3: 0Gy, n=7-8; 16Gy, n=8-9/ day 6: 0Gy, n=7-8; 16Gy, n=7/ day 15: 0Gy, n=7-8; 16Gy, n=8/ week 20: 0Gy, n=7; 16Gy, n=8-10. (Two-way ANOVA, Sidak test).

Supplementary figure S3. AMs CD11b- versus AMs CD11b+



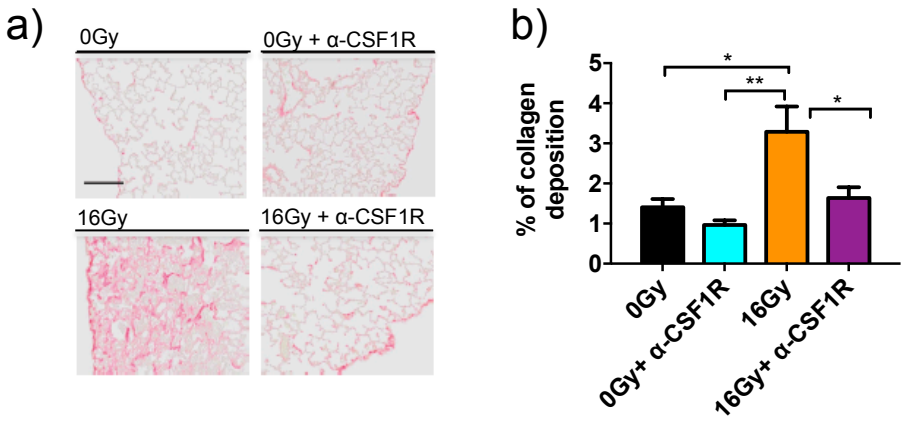
Supplementary Figure S4. Characterization of AMs and IMs 15 weeks post-IR. a) Percent of AMs in irradiated (16Gy) C57BL/6 mice (n=3) at 15 weeks post-IR. b) and c) Membrane expression of Icam1 and CD206 in AMs determined as the mean of fluorescence intensity (ΔMFI). d) Percent of IMs in irradiated (16Gy) C57BL/6 mice (n=3) at 15 weeks post-IR. e) and f) Percent of IMs expressing Icam1 and M2 IMs expressing CD206. Data were obtained from one experiment presented as ±SEM. *P<0.05; **P<0.005 (Student's *t*-test).

Supplementary figure S4. Thoracic IR increases pulmonary macrophage infiltration and their activation at 15 weeks post-IR



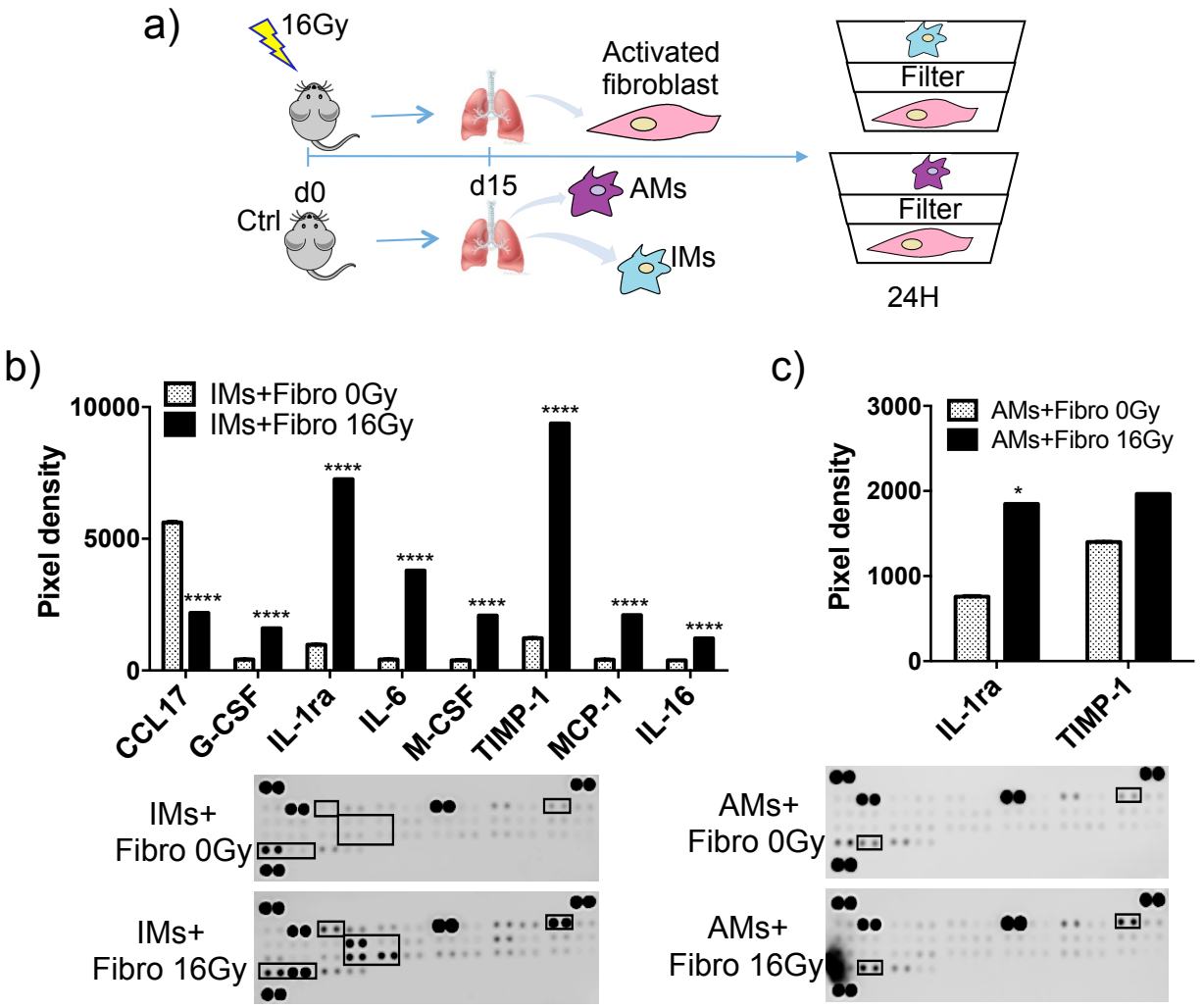
Supplementary figure S5. Fibrosis score assessment by Sirius Red staining after CSF1R inhibition. a) Sirius red staining of lung section from treated and non-treated C57BL/6 mice at 20 weeks post-IR. Scale bar: 100 μm. b) Lung fibrosis score quantification, presented as the percent of collagen deposition. Data were obtained from one experiment presented as mean±SEM, n=4 in each group. *P<0.05; **P<0.005 (One-way ANOVA, Tukey test).

Supplementary figure S5. Fibrosis score assessment by Sirius Red staining after CSF1R inhibition



Supplementary figure S6. Coculture of activated fibroblasts and non-activated macrophages. a) C57BL/6 mice (n=3) were irradiated at the thorax (16Gy) or mock irradiated. At 15 weeks post-IR, isolated lung tissue was digested. Activated and non-activated (Ctrl) fibroblasts were isolated from irradiated and non-irradiated mice respectively. AMs and IMs controls were isolated from non-irradiated aged-matched mice. Activated fibroblasts and control macrophages were cocultured for 24 hours (in triplicate). b) and c) Coculture supernatant was analyzed using cytokine arrays. Data were obtained from one experiment presented as mean±SEM, n=3 in each group. *P<0.05; ****P<0.00005 (Tow-way ANOVA, Sidak test).

Supplementary figure S6. activated fibroblast and non-activated macrophage co-culture



Supplementary figure S7. Thoracic IR procedure. Single dose of 16Gy was given locally at the whole thorax. To study acute response mice were sampled at 3, 6, 15 days post-IR, and to study late response to IR mice were sampled 20 weeks post-IR.

Supplementary figure S7. Thoracic IR procedure