SUPPLEMENTAL MATERIAL

Role for IL-1R1/MyD88 Signalling in Development and Progression of Pulmonary Hypertension

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"Take home" message: The IL-1R1/MyD88 pathway is a treatment target for pulmonary arterial hypertension.

SUPPLEMENTAL METHODS

Collection of human tissue samples

Lung tissue was obtained from 6 patients with iPAH who underwent lung transplantation at the Universitaire Ziekenhuizen in Leuven, Belgium. Table 1 in the online-only data supplement reports their haemodynamic and clinical characteristics. The collection protocol was approved by the Institutional Ethics Committee of the Leuven Universitaire Ziekenhuizen, and all participants gave written informed consent.

Control lung tissue was collected from 6 patients undergoing lung resection surgery for localised lung tumours at the Institut Mutualiste Montsouris (Paris, France). The controls had a >70% ratio of forced expiratory volume in the first second over forced vital capacity; none of the patients with iPAH or controls had chronic cardiovascular, hepatic, or renal disease or a history of cancer chemotherapy. The collection protocol was approved by the institutional review board of the Henri Mondor Teaching Hospital (Creteil, France), and all controls signed an informed consent document before study inclusion. Lung tissue was snap-frozen then stored at -80°C until use.

Studies in mice

Adult male mice 3 months of age (C57Bl/6j) were used according to institutional guidelines, which complied with national and international regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee of the French National Institute of Health and Medical Research (INSERM)–Unit 955, Creteil, France. Transgenic mice constitutively deleted for IL-1R1 (*IL-1R1*$^{-/-}$) or MyD88 (*MyD88*$^{-/-}$) were obtained from Dr R Flavell and Dr A Akira [1, 2]. Mice with *MyD88* gene deletion confined to the myeloid-cell lineage (M.lys-Cre MyD88$^{fl/fl}$ mice) were generated on BL6 ES

cells at the CNRS, UMR7355, Orleans, France, as described previously (A Maillard et al., submitted). Transgenic mice with 5-HTT overexpression in SMCs under the control of the SM22 promoter (SM22-5HTT^{+}) were produced and bred as previously described. SM22-5HTT^{+} mice are fertile and have a normal life span and normal growth but spontaneously develop PH [3]. The control animals were WT C57BL/6j mice obtained from Janvier (Le Genest-Saint-Isle, France). The IL-1R1 inhibitor anakinra (Kineret®, Amgen, Thousand Oaks, CA, USA) was administered daily by intraperitoneal injection in a dosage of 20 mg/kg[4]. Six to eight mice were usually studied for each treatment condition. At treatment completion, the lungs were removed and prepared for histological or Western blot analyses. All animals were housed in a room at constant temperature with a 12-hour light-dark cycle and fed ad libitum. Mice were exposed to chronic hypoxia (9% O_2) in a ventilated chamber (Biospherix, Lacona, NY, USA). The hypoxic environment was established by flushing the chamber with a mixture of room air and nitrogen then recirculating the gas mixture. The chamber was opened daily for 1 hour if drug treatment was necessary and twice a week otherwise to replenish the food and water supplies and to clean the cages. Normoxic mice were kept under similar conditions with the same light-dark cycle.

Assessment of pulmonary hypertension (PH) in mice

At the specified time points after hypoxia exposure, the mice were anaesthetised with intraperitoneal ketamine (60 mg/kg) and xylazine (10 mg/kg). After incision of the abdomen, a 26-gauge needle connected to a pressure transducer was inserted into the right ventricle through the diaphragm, and right ventricular systolic pressure (RVSP) was recorded immediately. Then, the thorax was opened and the lungs and heart were removed. The right ventricle (RV) was dissected from the left ventricle plus septum (LV+S), and these dissected samples were weighed for determination of Fulton's index (RV/[LV+S]). The lungs were

fixed by intratracheal infusion of 4% aqueous buffered formalin. A mid-sagittal slice of the right lung was processed for paraffin embedding. Sections 5 µm in thickness were cut and stained with hematoxylin-phloxine-saffron for examination by light microscopy. In each mouse, 30 to 40 vessels were examined by an observer who was blinded to the treatment or genotype. Each vessel was categorised as non-muscular (no evidence of vessel wall muscularisation) or muscular (partially muscular, over less than three-fourths of the vessel circumference; or fully muscular). The percentage of muscular pulmonary vessels was determined by dividing the number of partially or fully muscular vessels by the total number of vessels in the relevant group of animals. Dividing (Ki67+) vascular cells were identified in distal pulmonary vessel walls and expressed as the number of stained nuclei over the total number of nuclei counted in the media of at least 20 vessels per mouse.

Studies in rats

Experiments were performed in adult male Wistar rats (200 to 250 g). PH was induced by administering 60 mg/kg monocrotaline (Sigma-Aldrich, Lyon, France) subcutaneously. To assess the potential curative effects of anakinra, rats given monocrotaline were left untreated for 21 days then randomly assigned to treatment with anakinra (20 mg/Kg/d) or vehicle from day 21 to 42 (7-8 animals in each group), given intraperitoneally once a day. Rats in each group were euthanised on days 21 and 42 for assessments of PH. After anaesthesia with ketamine (60 mg/kg IM) and xylazine (3 mg/kg IM), a polyvinyl catheter was introduced into the right jugular vein and pushed through the right ventricle into the pulmonary artery for the measurement of pulmonary arterial pressure (PAP). The thorax was then opened and the lung immediately removed. The heart was dissected and weighed for calculation of Fulton's index (RV/[LV+S]). The lung was fixed in the distended state with formalin buffer. After paraffin embedding, 5 µm-thick lung sections were stained with

haematoxylin-phloxine-saffron. In each rat, the distribution and degree of artery muscularisation were assessed by categorising 40 to 60 intra-acinar arteries as muscular or non-muscular. Normalised arterial wall thickness was calculated as the ratio of the difference between the external and internal diameters over the external diameter of the pulmonary artery.

Macrophage isolation and polarisation

Macrophages were prepared from bone marrow cultures. Bone marrow was isolated from the femur and tibia as previously described [5]. Briefly, all muscle tissue was removed from the bones, which were washed in 70% alcohol for 5 seconds then washed twice in phosphate-buffered saline (PBS). The ends of the bones were cut with scissors and the marrow harvested by flushing with PBS using a syringe and 22-gauge needle. The PBS containing bone marrow was passed through a 100-μm mesh and centrifuged at 500 *g* in RPMI-1640 with 10% foetal calf serum (FCS). Red blood cells were lysed using ACK lysis buffer on ice, for 2 minutes. Isolated bone marrow cells were cultured in RPMI-1640 with 10% FCS and 50 ng/mL of macrophage colony-stimulating factor (M-CSF), for 7 days, in 6-well plates. On day 6, we added 10 ng/mL of lipopolysaccharide and 50 ng/mL of interferon-γ to obtain M1 differentiation, 20 ng/mL of IL-4 to obtain M2 differentiation, or RPMI-1640 to obtain M0 (undifferentiated) macrophages [6]. On day 7, the non-adherent cells were discarded.

Conditioned media preparation

Undifferentiated macrophages (M0) and M1 and M2 differentiated macrophages were obtained from WT mice and MyD88$^{-/-}$ mice (see previous section). Cell viability as assessed by trypan blue exclusion was greater than 85% in all groups. To produce medium-

conditioned cells, we replaced the medium by serum-free RPMI-1640 for 48 hours. For the PA-SMC proliferation assay, macrophage-conditioned media were diluted 2-fold with fresh serum-free medium and applied for 48 hours to PA-SMCs [7], which were pre-treated by anakinra (5 µM) or vehicle during 24 hours.

Pulmonary-artery smooth muscle cell (PA-SMC) proliferation

PA-SMCs from human or mouse pulmonary arteries were cultured and characterised as previously described [8]. After 24 hours' incubation in Dulbecco's modified Eagles medium containing 0.1% serum, the cells were treated with 15% FCS, platelet-derived growth factor (PDGF)-BB (40 ng/mL), IL-1ß (50 ng/mL), or HMGB1 (20-50 ng/mL). Anakinra (Kineret®) 5 µM or vehicle was added in serum-free medium for 24 hours before growth-factor exposure. After 48 hours, 0.2 mg/mL tetrazolium salt (MTT, Sigma-Aldrich, St. Louis, MO, USA) was added to each well and left for 6 hours. The culture medium was then removed and formazan crystals were solubilised by adding 100 µL dimethyl sulphoxide. The reduction of tetrazolium salt to formazan within the cells was quantified by spectrophotometry at 520 nm and taken to reflect the cell count.

To confirm the effects of IL-1ß and anakinra on PA-SMC proliferation, PA-SMCs were exposed to anakinra (5 µM) or vehicle in serum-free medium then to IL-1ß (50 ng/mL). Then, the cells were incubated with a BrdU-labelling reagent (final concentration, 10 µmoL/L) for 48 hours before detection of BrdU-labelled cells (BrdU Labelling and Detection Kit I, Roche, Penzberg, Germany). BrdU incorporation was quantified by spectro-photometry at 370 nm (with the reference wavelength at 492 nm), expressed in optical density units, and taken as an indicator of the cell count. BrdU values were usually obtained from 16 to 24 wells of cells from independent experiments.

Immunohistochemistry

Paraffin-embedded sections were deparaffinised using xylene and a graded series of ethanol dilutions then incubated in citrate buffer (0.01 M, pH 6) at 90°C for 15 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 and 10% methanol in PBS for 10 minutes. Permeabilisation was achieved using 0.1% Triton X-100 in PBS for 10 min. The slides were incubated for 60 minutes in 1% bovine serum albumin and 5% goat serum in PBS then incubated overnight with the primary antibody anti-Ki67 (1:200, Abcam, Cambridge, UK). We used horseradish peroxidase-(HRP) conjugated anti-rabbit (1:200, Dako, Glostrup, Denmark) as the secondary antibody and DAB (FastDAB, Sigma-Aldrich) for substrate staining. The sections were counterstained with hematoxylin to identify dividing Ki67+ vascular cells.

For immunofluorescence, lung tissue slides were incubated overnight at 4°C with anti-IL-1R1 (1:100, Abcam), anti-MyD88 (1:200, Abcam), anti-α-smooth muscle actin (αSMA) rabbit antibody (1:400, Abcam), or anti-F4/80 (1:50, Serotec, Oxford, UK). They were then exposed to anti-mouse Alexa Fluor® 488 and anti-rabbit Alexa Fluor® 555 (1:1000, Cell Signaling Technology, Beverly, MA, USA) antibodies. Nuclei were stained with Hoechst (1 µL/mL, Cell Signaling Technology). Fluorescence was measured using an Axioimager M2 Imaging microscope (Zeiss, Oberkochen, Germany) and quantified on digital photographs using Image J software (imagej.nih.gov/ij/).

Cell immunostaining

PA-SMCs were cultured on Lab-Tek™ (Thermo Scientific, Waltham, MA, USA; 10 000 cells/well), fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton-X100, and immunostained with the indicated primary antibodies. Visualisation was achieved using the appropriate Alexa Fluor®-conjugated antibody (see previous section).

Real-time quantitative PCR (RT-qPCR)

Total mRNA was extracted from lung-tissue specimens each weighing 20 mg and stored at -80°C, using RNeasy Protect Mini Kit (Qiagen, ZA Courtaboeuf, France). First-strand cDNA was synthesised in reverse transcribed samples, as follows: 1 µg total RNA isolated from cells, 100 ng Random Primers, 0.4 mM mixed dNTP, 40 U RNaseOUT, and 200 U Superscript II. All reagents were obtained from Life Technology (Carlsbad, CA, USA). Quantitative PCR was performed using a QuantStudio™ 6 Flex Real-Time PCR System with SYBR Green (both from Life Technology).

Specific primers were as follows: mouse IL-1ß, Fwd 5'-CATGAGCACCTTCTTTTCCTTCA-3', Rev 5'-CACACACCAGCAGGTTATCATCA-3'; mouse IL-1R1, Fwd 5'-TCCGGTCACACGAGTAATAC AATT-3', Rev 5'-GGGCTCAGGATAACAGGTCTGT-3'; mouse MyD88, Fwd 5'-GAGGCAT CACCACCCTTGA-3', Rev 5'- GCAGTAGCAGATAAAGGCATCGA-3'; and mouse IL-6, Fwd 5'- CTCTGGGAAATCGTGGAAATG -3', Rev 5'- AAGTGCATCATC GTTGTTCATACA -3'; rat IL-1ß, Fwd 5'-TCCATGAGCTTTGTACAAGG-3', Rev 5'-GGTGCTGATGTACCAGTTGG-3'; rat IL-6, Fwd 5'-TGTTCTCAGGGAGATCTTGG-3', Rev 5'-TCCAGGTAGAAACGGAACTC-3'; rat 18s, Fwd 5'-TTGATTAAGTCCCT GCCCTTTGT-3', Rev 5'-CGATCCGAGGGCCTAACTA-3'. To normalise for cDNA input load, mouse 18s was used as the endogenous standard. The analysis was performed using the standard $^{\Delta\Delta}$Ct method.

Protein extraction

Lung-tissue specimens and PA-SMCs were defrosted and homogenised in a Tissue Lyser® homogeniser (Qiagen), using 200 µL of ice-cold RIPA buffer containing protease and phosphatase inhibitor (Sigma Aldrich) for Western blotting, or using 200 µL of T-PER®

tissue-protein extraction reagent (Thermo Scientific Pierce, Illkirch, France) for ELISA. The homogenate was centrifuged at 10 000 *g* for 5 minutes to remove tissue debris, and the supernatant containing the total lung lysate was used for Western blotting or ELISA. The total protein level in lysate was quantified using the Biorad DC protein assay (Biorad, Marnes-la-Coquette, France).

Western blot and ELISA

Briefly, separated proteins were transferred to nitrocellulose membranes, blocked for non-specific protein binding using TBS-Tween in 0.1% and 5% milk, and probed for selected proteins using a specific primary antibody followed by an HRP-conjugated secondary antibody (1:1000, GE Healthcare, Little Chalfont, UK) for detection. The primary antibodies were against IL-1R1 (1:200, Abcam), MyD88 (1:500, Abcam), phosphorylated NF-κB p65 Ser536 (1:200, Abcam), NF-κB p65 (1:200, Abcam), ASC, caspase-1 (1:1000, Santa Cruz Biotech, Dallas, TX, USA), IL-18 (1:500, Santa Cruz Biotech), and β-actin (1:10 000, Sigma-Aldrich). After overnight incubation with the primary antibody at 4°C, the samples were incubated with the corresponding HRP-conjugated secondary antibody (1:1000, GE Healthcare). Densitometric quantification was normalised to β-actin in each sample (Gene Tools, Ozyme, Montigny Le Bretonneux, France).

All ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA) and used according to manufacturer guidelines. Before each ELISA, various dilutions of protein lysates were tested to ensure that the detected level fit within the standard linear range.

Statistical analysis

Quantitative variables from human and animal studies are reported as mean and individual values. Data from quantitative polymerase chain reactions, Western blotting, and

ELISA are reported as mean±SEM. These variables were compared using the Mann-Whitney post hoc test with the Bonferroni correction for multiple comparisons. Cell-study results are reported as mean±SEM and compared using standard one-way ANOVA with Sidak's multiple comparison test (Prism 6.0, GraphPad Software, San Diego, CA, USA). Values of $p < 0.05$ were considered significant.

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SUPPLEMENTAL FIGURE LEGENDS:

Supplemental figure 1. Immunolocalization of IL-1R1 and MyD88 in WT, IL-1R1 and MyD88 deficient mice. Representative micrographs of lung tissue from WT mice, IL-1R1$^{-/-}$ and MyD88$^{-/-}$ mice. IL-1R1 or MyD88 (red), α-smooth muscle actin (α-SMA; green) for SMC staining, or Hoechst for nucleus staining (blue). No immunoreactivity was detected in sections incubated with rabbit IgG control and secondary anti-rabbit antibody. Bar, 30 µm.

Supplemental figure 2. The IL-1R1/MyD88 pathway is involved in the spontaneous development of pulmonary arterial hypertension (PAH) in SM22-5HTT^{+} mice characterized by 5-HTT overexpression in SMCs under the control of the SM22 promoter.

A, IL-1R1, MyD88, IL-1ß, and IL-6 mRNA levels measured with quantitative real-time polymerase chain reaction in lungs from wild-type (WT) and SM22-5HTT^{+} mice. Data are mean±SEM of 8 animals

B, Graphs of right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (right ventricle/left ventricle plus septum weight [RV/(LV+S)]), pulmonary vessel muscularisation, and dividing Ki67+ cells in WT mice and SM22-5HTT^{+} mice treated daily for 21 days with vehicle or anakinra (100 mg/kg). Representative micrographs of pulmonary vessels stained for Ki67. Red arrows show Ki67+ nuclei. No immunoreactivity was detected in sections incubated with rabbit IgG control and secondary anti-rabbit antibody. Bar, 50 µm. Data are mean±SEM of 6 animals. ***p<0.005 compared with values in control WT mice; $^{\#\#}p$<0.01

Supplemental figure 3. Lung protein levels of Phosphorylated NF-κB p65, ASC, caspase-1 and IL-18 protein levels in mice exposed to chronic hypoxia.

A, ASC, caspase-1 activity, IL-18 and ratio of phosphorylated NF-κB p65 Ser536 (p-NF-κB) on NF-κB p65 proteins levels measured by Western blot in lungs from wild-type (WT) mice after hypoxia exposure for various durations. The Caspase-1 activity is represented by the ratio caspase-1/pro-caspase-1.

B, Ratio of phosphorylated NF-κB p65 Ser536 (p-NF-κB) on NF-κB p65 proteins, ASC, caspase-1 activity and IL-18 protein levels measured by Western blot in lungs from wild-type (WT), IL-1R1 KO and MyD88 KO mice after 21 days of hypoxia exposure. Data are mean±SEM of 6 animals.

C, activation of NF-κB, expressed as the ratio of phosphorylated NF-κB p65 Ser536 (p-NF-κB) over NF-κB p65 proteins levels, measured by Western blot in lungs from WT mice exposed to hypoxia during 12 hours (Hx 12 h) or 21 days (Hx 21 d), and treated daily with anakinra (20 mg/kg) or vehicle. *p<0.05, **p<0.01, compared with values in control mice exposed to normoxia. $^{\#}p$<0.01, compared with value in controls WT mice exposed to hypoxia.

Supplemental figure 4. Proliferation of PA-SMCs from wild-type (WT) mice stimulated by IL-1ß (50 ng/mL) or 15% foetal calf serum (FCS) with or without anakinra (5 µM). Data are mean±SEM of 18-24 values from at least two different experiments. ***p<0.005 compared to control cells.

Supplemental figure 5. Effects of IL-1R1 activation by IL-1ß stimulation and TLR-4 activation by HMGB1 stimulation on proliferation of pulmonary-artery smooth muscle cells (PA-SMCs).

A, Proliferation of human PA-SMCs stimulated by different doses of IL-1ß (5, 10, or 50 ng/mL) with or without pretreatment with anakinra (5 µM). Data are mean±SEM of 14

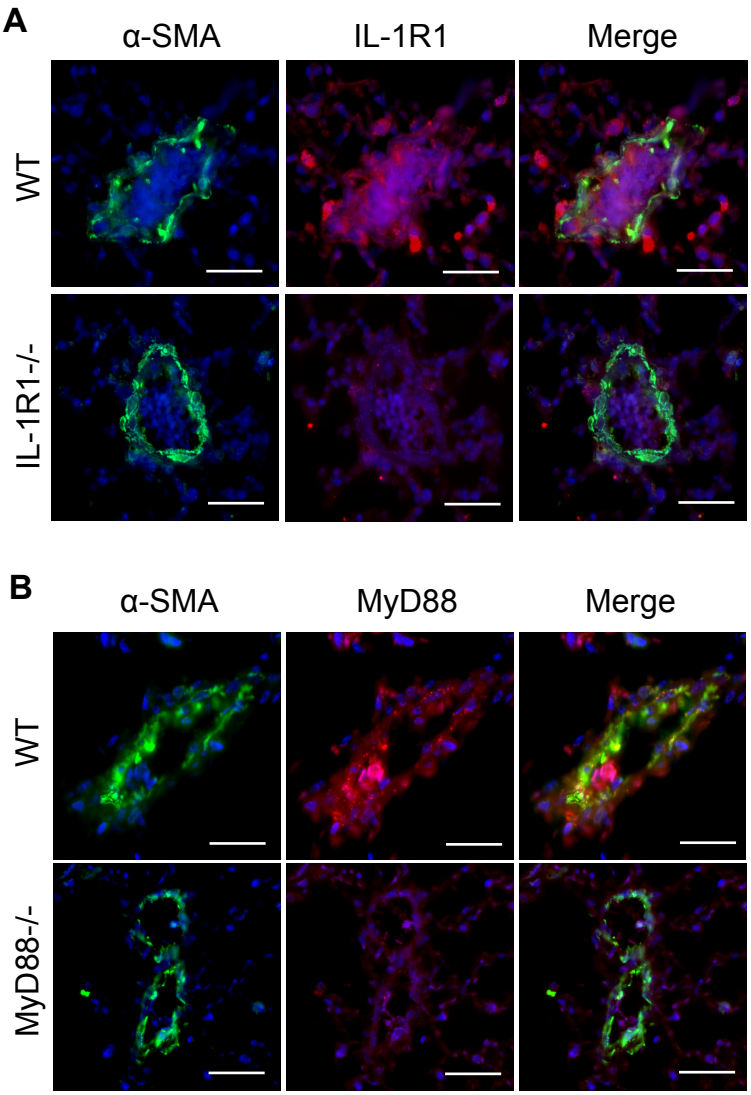
values from at least two different experiments. **p<0.0033, ***p<0.0016 compared to control cells.

B, Proliferation of mice PA-SMC stimulated by different doses of HMGB1 (25 or 50 ng/mL) with or without foetal calf serum (FCS, 5% or 15 M). Data are mean±SEM of 18 values from at least three different experiments. *p<0.05 compared to PA-SMCs treated with vehicle. OD, optical density.

Supplemental figure 6. NF-κB activation, expressed as the ratio of phosphorylated NF-κB p65 Ser536 (p-NF-κB) on NF-κB p65 proteins, ASC, caspase-1 and IL-18 protein levels measured by Western blot in PA-SMCs from normoxia (Nx mice) or hypoxia mice (Hx mice). Cells were stimulated 48 hours by IL-1ß (50 ng/mL) with or without anakinra pretreatment (5 µM). The Caspase-1 activity is represented by the ratio caspase-1/pro-caspase-1. Data are mean±SEM of PA-SMC from 6 different animals. *p<0.05, **p<0.01 compared to PA-SMCs treated with vehicle. $^{\#}p$<0.05.

Supplemental figure 7. NF-κB activation, expressed as the ratio of phosphorylated NF-κB p65 Ser536 (p-NF-κB) on NF-κB p65 proteins, and IL-1ß and IL-6 mRNA levels in lungs from rats studied at day 42 after monocrotaline administration and treated from day 21 to day 42 with either vehicle or anakinra. $^{\#}$p<0.05 compared with values in vehicle treated rats.

Supplemental figure 1



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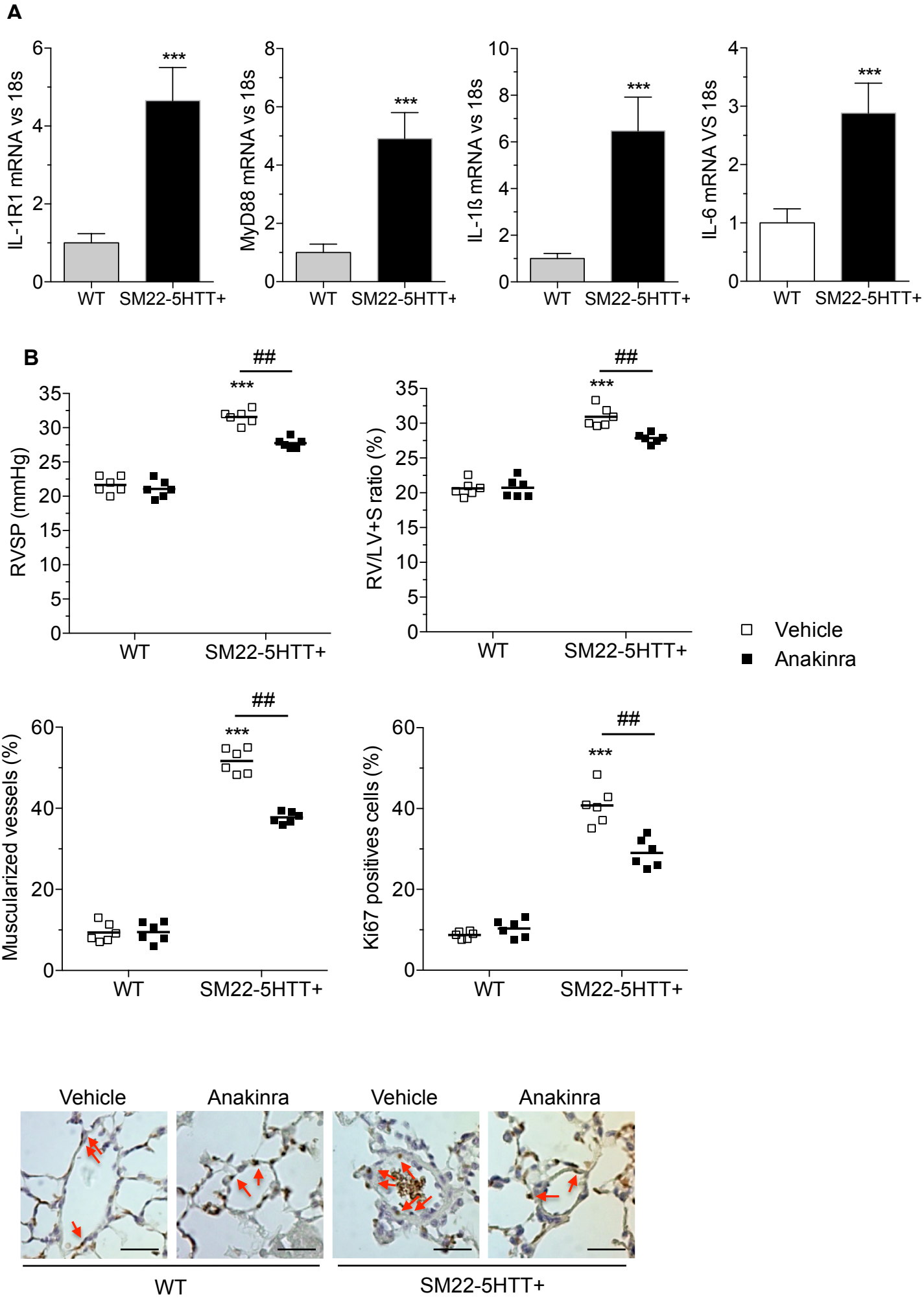

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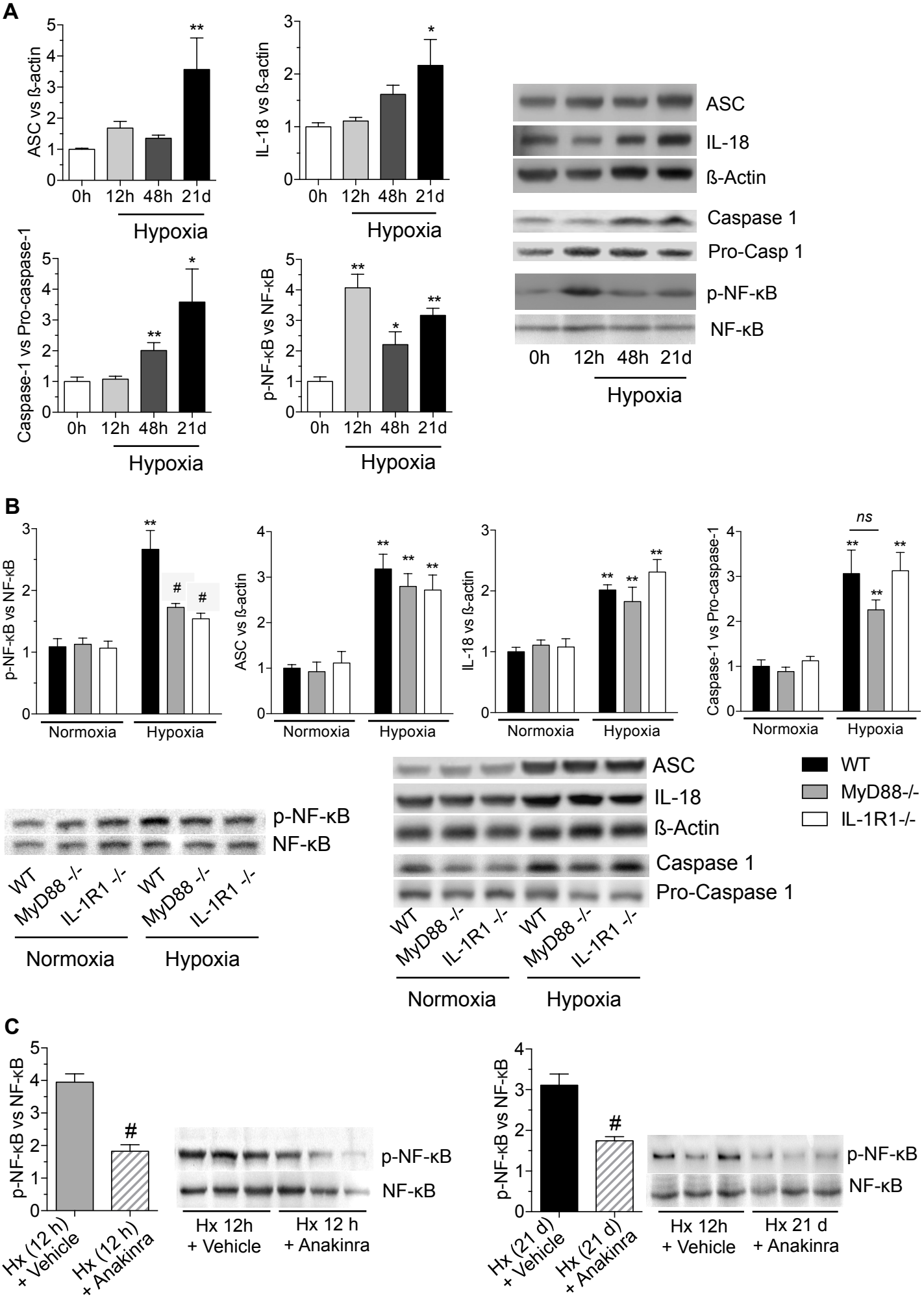

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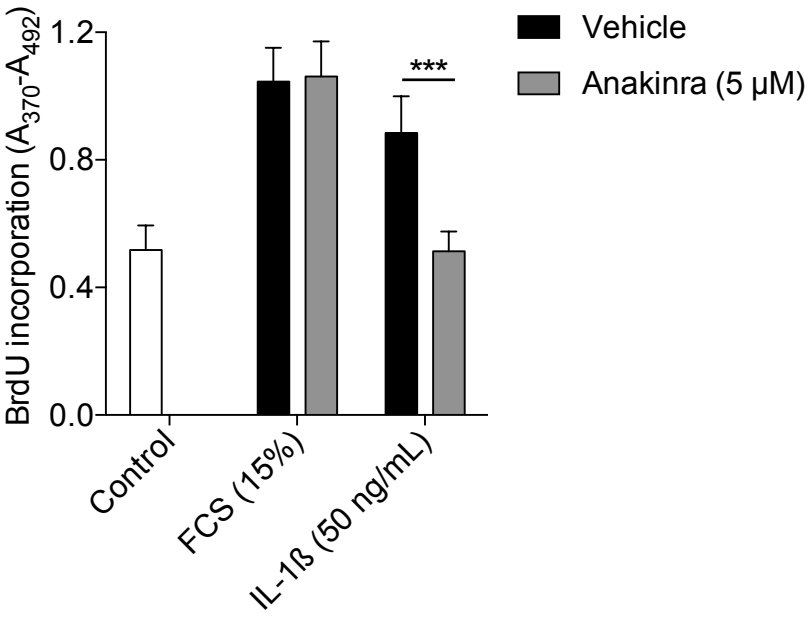

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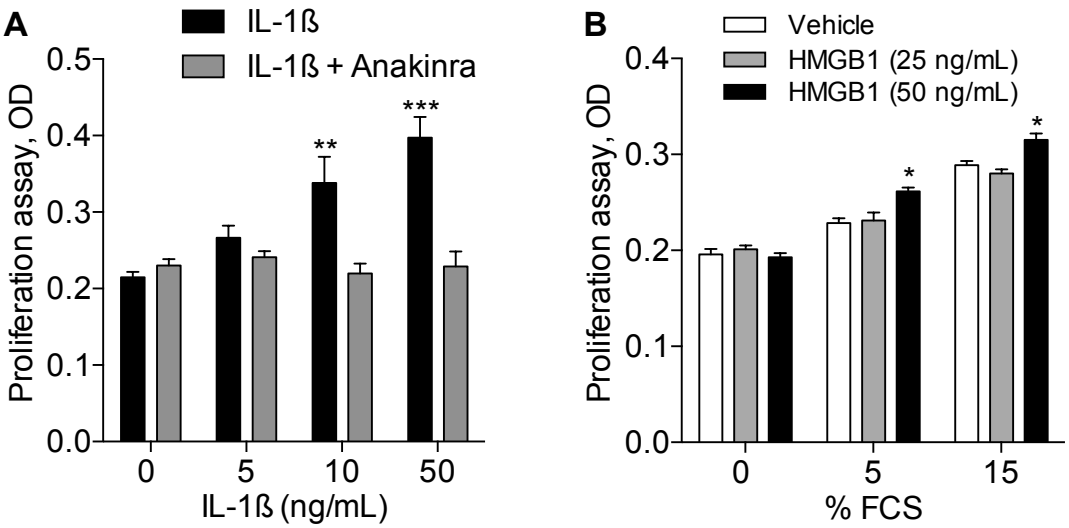

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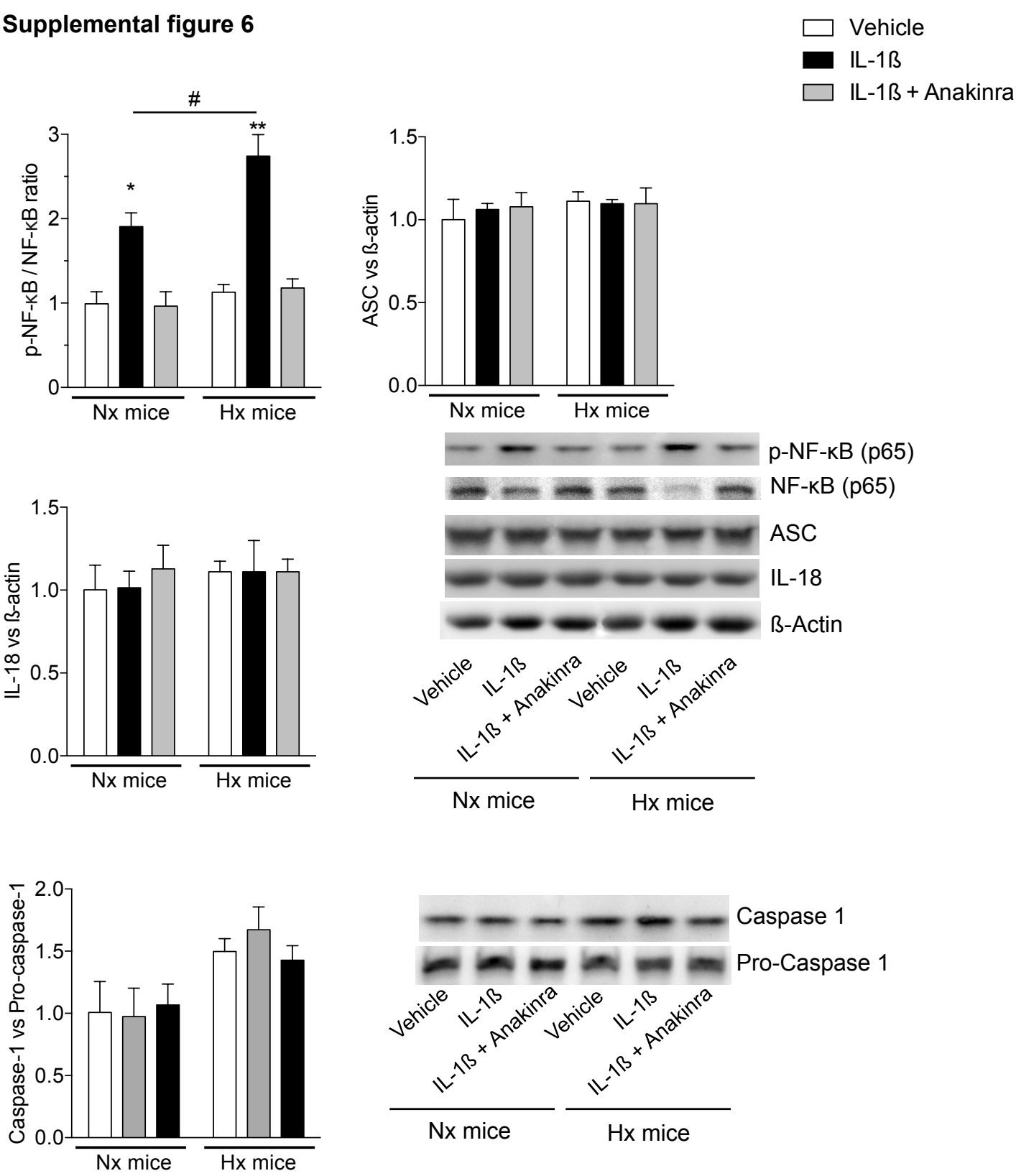

Supplemental figure 7