Supplemental file 1

Yue X, *et al. Ann Rheum Dis* 2022;0:1–9. doi: 10.1136/annrheumdis-2021-222088

Materials and methods

Mice

Female wild type C57BL/6J mice, $CD4^+$ T cell-deficient mice (B6.129S2-$Cd4^{tm1Mak}$/J, CD4 KO), $CD8^+$ T cell-deficient mice (B6.129S2-$Cd8a^{tm1Mak}$/J, CD8 KO) and B cell-deficient mice (B6.129S2-$Ighm^{tm1Cgn}$/J, muMT KO) were purchased from the Jackson Laboratory (USA) via Charles River Laboratories (Germany). AT1R-deficient mice (B6.129P2-$Agtr1a^{tm1Unc}$/$Agtr1b^{tm1Cof}$/J, AT1R KO) and their littermate controls were kindly provided by Prof. Thomas Walther (University of Hull, UK) and bred at the animal facility of the Research Center Borstel. All mice were housed under specific pathogen-free conditions with 12-hour light/darkness cycles in the animal facility at the Research Center Borstel or at Xiamen University in China. Animal studies comply with the Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines and have been carried out according to European Union Directive 2010/63/EU. All studies were reviewed and approved by the Animal Research Ethics Board of the Ministry of Energy, Agriculture, the Environment, Nature and Digitalization, Kiel, Germany (reference number V241-47120/2017) or by the Institutional Animal Care and Use Committee of Xiamen University.

Immunization

Eight- to nine-week-old female C57BL/6J mice were immunized with 0.2 mg of ME prepared from CHO cells overexpressing human AT1R (hAT1R; CellTrend, Germany) in 50 µl of PBS emulsified with an equal volume of complete Freund adjuvant (CFA; Sigma-Aldrich, USA) by subcutaneous injection into the footpad. Three weeks after the primary immunization, the mice were boosted with the same amount of hAT1R-containing ME emulsified with incomplete Freund adjuvant (IFA). In the control group, mice were treated with the same amount of ME from un-transfected CHO cells (CellTrend, Germany) according to the same immunization protocol. Six weeks after the

booster immunization, all mice were sacrificed for sample collection and disease symptom evaluation. For the immunization with AT1R_149-172 peptide, CIIIWLLAGLASLPAIIHRNVFFI, Female C57BL/6J mice were immunized with 0.2 mg of AT1R_149-172 peptide or control solvent emulsified with an equal volume of complete Freund adjuvant by subcutaneous injection into the footpad. Two weeks after the first immunization, the mice were boosted with the same amount of antigen emulsified with incomplete Freund adjuvant (IFA). Three weeks after the first immunization, mice were sacrificed for further evaluation.

Differential leukocyte counts in bronchoalveolar lavage fluid

Immediately after sacrificing the mice by CO_2 asphyxiation, bronchoalveolar lavage fluid (BALF) was collected, and the total number of cells was counted with a hemocytometer. After centrifugation at 500 *g* for 5 min, BALF cell pellets were resuspended in PBS containing 30% fetal bovine serum (FBS; PAN biotech, Germany). The cell suspensions were spun down onto slides and stained with a Giemsa May-Grünwald solution (Sigma-Aldrich, USA). Differential leukocyte counts were calculated from 300 total cells for each sample.

Purification of serum IgG

IgG in serum from mice sacrificed at week 9 after immunization was purified by affinity chromatography with a HiTrap protein G Affinity column (GE Healthcare, USA). The IgG fractions were eluted with 0.1 M glycine HCl buffer (pH 2.7), which was subsequently neutralized with 1 M Tris-HCl buffer (pH 9.0). The concentration of IgG in the preparations was determined photometrically at a wavelength of 280 nm (Nanodrop1000 spectrophotometer, Thermo Fisher Scientific, USA).

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Histological assessment

Skin and lung samples were fixed in 4% formalin for 24 hours, embedded in paraffin, and cut into 3-μm-thick sections. To evaluate histopathological changes and fibrosis, the paraffin-embedded sections were subjected to hematoxylin and eosin (H&E) staining (Roth, Germany) or Masson trichrome staining (Sigma-Aldrich, USA). The inflammatory score was calculated based on the number and area of focal infiltrates or number of affected vessels. Briefly, all tissue sections were inspected first for the average area of single focal infiltrate or perivascular infiltrate. In each field of each section, the infiltrates with average size were scored as 1, while the infiltrate with smaller and larger size were scored as 0.5 and 1.5, respectively. For each section, the inflammatory score was defined as the sum of the scores of all fields. Scoring was performed by two investigators in a blinded manner. Skin thickness was measured as the thickness of the collagen layer.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on skin and lung sections after formalin fixation followed by paraffin embedding. Briefly, tissue sections were deparaffinized in xylol and rehydrated in a gradient of ethanol solutions. Antigen retrieval was performed by heating the slides in 10 mM citrate buffer (pH 6.0) for 1 hour, and endogenous peroxidase activity and nonspecific binding were blocked with 3% H_2O_2 and 5% Bovine Serum Albumin (BSA) solutions, respectively. The sections were incubated overnight at 4°C with rabbit anti-CD3 Abs (polyclone, Abcam, Hongkong), CD45R (RA3-6B2, eBioscience, USA), or neutrophil marker (Biotin clone 7/4, Canada). Then, the sections were incubated with an appropriate biotinylated goat polyclonal secondary antibody directed against rabbit IgG (Jackson ImmunoResearch, USA) for 45 min at room temperature (RT), followed by incubation in an avidin biotinylated-HRP solution (Vector, USA) for 20 min at RT. Diaminobenzidine (Vector Laboratories, USA) was applied to visualize

immunoreactivity. Afterwards, the sections were counterstained with hematoxylin for 5 min. Images were acquired by bright-field microscopy at a magnification indicated in the figure legends (Nikon, Japan).

Immunofluorescence (IF) staining

The deposition of IgG derived from murine serum by human epithelial type 2 (HEp-2) cells was determined using indirect IF*. As shown before,[24] these cells express membranous AT1R. First,* HEp-2 cells were incubated with IgG isolated from hAT1R-immunized mice or control-immunized mice purified by affinity chromatography on Protein G Sepharose at a concentration of 20 µg/ml for 30 min at RT, and IgG deposition was detected using a DyLight 649-labeled goat anti-mouse IgG antibody (polyclonal, BioLegend, USA). Similarly, IgG deposition in murine lung tissue was detected using the same secondary ab. In addition, myofibroblasts and activation of Smad signaling in murine skin were also determined using IF staining. Briefly, skin cryosections of from hAT1R-immunized mice or control mice were incubated with a mouse anti-mouse α-SMA antibody and a Dylight 649-conjugated goat anti-mouse antibody to detect myofibroblasts, a goat anti-mouse p-Smad2/3 antibody and Alexa 488-conjugated donkey anti-goat IgG antibody was applied to detect p-Smad2/3 positive cells. Coverslips were mounted with Gold mounting agent (Thermo Fisher Scientific, USA) that contains 4`, 6-diamidino-2-phenylindole (DAPI) for nuclear staining, the fluorescence was evaluated by confocal microscopy at a magnification indicated in the figure legends (Leica SP5, Germany).

Detection of apoptotic endothelial cells

After fixation in a 4% pre-chilled paraformaldehyde solution and blocking with a 5% BSA solution, cryosections of lung samples were incubated with a rat anti-mouse CD31 antibody (clone 390,

BioLegend, USA) for 45 min at RT and then an Alexa Fluor 546-conjugated goat anti-rat IgG (polyclonal, Thermo Fisher Scientific, USA). After the endothelial cells were labeled, apoptotic cells were detected with a TUNEL kit (Promega, USA) according to the product manual. Briefly, the sections were permeabilized with 0.5% Triton X-100 and equilibrated in 100 µl of equilibration buffer. Immediately before the reaction, the TUNEL mixture was freshly prepared and added to the sections. After incubation at 37°C for 60 min, the sections were immersed in 2x SSC buffer to stop the reaction. Coverslips were mounted with Gold mounting agent (Thermo Fisher Scientific, USA), and fluorescence was detected by confocal microscopy (Leica SP5, Germany). To quantify the level of apoptotic endothelial cells, 10 random fields with vessels were acquired for each lung sample, and the numbers of apoptotic endothelial cells and total endothelial cells were counted. The level of apoptotic endothelial cells is presented as the ratio of apoptotic to total endothelial cells.

Determination of collagen content

To assess the collagen content in tissues, 0.25 mm^2 samples of tissue from the skin or the postcaval lobe of the lungs were homogenized in a 0.5 M acetic acid solution containing 0.1 mg/ml porcine pepsin and incubated at 4°C overnight. The pepsin-soluble collagen content was collected after removal of insoluble debris by centrifugation at 12,000 *g* for 10 min at 4°C. The amount of extracted collagen was measured using a Sircol collagen detection kit (Biocolor, UK).

Enzyme-linked immunosorbent assay (ELISA)

ELISA plates coated with ME from CHO cells overexpressing hAT1R (CellTrend, Germany) were used to detect the levels of AT1R Abs and the Ab subclasses in mouse serum samples. First, the ELISA plates were incubated with serum samples diluted at log intervals (1:10^2 to 1:10^7). After

incubation with an HRP-conjugated goat anti-mouse IgG antibody, or HRP-conjugated goat anti-mouse IgG subclass (IgG1, IgG2a, IgG2b, IgG3) Abs (Jackson ImmunoResearch, USA) at RT for 1 hour, 3,3',5,5'-tetramethylbenzidine (TMB) was applied to visualize the signal. Optical density (OD) values were measured at 450 nm on a microreader (Tecan Life Science, Switzerland) and plotted against dilution (log scale). The levels of AT1R Abs and Ab subclasses were defined as the dilution at which the OD value reached half of the maximal OD values of the curve. CCL18 in supernatants were measured using a DuoSet ELISA (R&D Systems, Germany) according to the manufacturer's instructions. Absorbance was measured using Mark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Germany).

Monoclonal Ab specific for AT1R

A monoclonal Ab against hAT1R was generated from hAT1R-immunized mice using the conventional hybridoma technique. Unlike the mice immunized according to the immunization protocol described above, the mice in this experiment received an additional booster immunization on day 34 and were sacrificed on day 36. Lymphocytes derived from the spleen and draining lymph nodes were fused with mouse myeloma cells (Ag8.653), and culture supernatants were screened for AT1R binding by ELISA with slight modifications.[11] After 7 rounds of limiting dilutions, a clone producing an AT1R Ab of the IgG2a subclass (clone 5.2a) was designated as monoclonal. This mAb was sequenced, recombinantly expressed in HEK293 cells, purified and subsequently used for *in vitro* experiments.

Cardiomyocyte beating assay

Cardiomyocytes were prepared from the hearts of 1- to 3-day-old neonatal rats as described in detail by Wallukat *et al*. [23]. Briefly, the cells were cultured as a monolayer for 4 to 10 days at 37°C

in SM20-I medium supplemented with 10% heat-inactivated neonatal calf serum and 2 μM fluorodeoxyuridine. To determine the effect of AT1R Abs on cardiomyocytes, spontaneously beating cultured cells were treated with IgG purified from immunized mice or mAT1R Ab (clone 5.2a). The specificity of the effect was confirmed by using the AT1R antagonist losartan (Sigma-Aldrich). The beating rate of the cells was measured on the heated stage (37°C) of an inverted microscope, and the basal beating rate was determined in 6 fields specified by marks on the bottom of the culture flask. One hour after treatment with Abs, the beating rates of the cardiomyocytes in the marked fields were re-measured, and the results are expressed as an increase/decrease in the number of beats per minute. Personal was not aware of the IgG source.

Transfer of anti-AT1R IgG

The concentration of murine IgG purified from immunized mice was diluted to 1 μg/μl in PBS, and 50 μg of IgG was injected intradermally (i.d.) into one mouse ear. After 24 hours, ear samples were collected for histological assessment by H&E staining. Alternatively, mAT1R Ab (clone mAb5.2a) or an Ab of the same isotype (BioLegend, USA) was injected i.d. into the ear repeatedly every other day from day 0 to day 12 (100 μg of IgG/injection). On day 14, the mice were sacrificed, and inflammation in the antibody-treated ear and the lungs was evaluated histologically.

Human monocyte isolation

Monocytes were derived from peripheral blood mononuclear cells (PBMC) from healthy donors (n=6) after written informed consent using guidelines approved by the Ethics Committee of the University Hospital of Luebeck, 16-199). Monocytes were isolated after density-gradient centrifugation, subsequent plastic adherence for 2h and cultured in RPMI 1640 supplemented with

10% fetal calf serum (Bio&SELL, Germany) and 1% penicillin/streptomycin (Thermo Fisher Scientific, USA).

Monocyte assay

Primary human peripheral blood monocytes of healthy donors were used in two assays (n=3 each) and stimulated with the AT1R ligand Ang II (5 µM), the anti-human mAT1R Ab (clone 5.2a, 100 or 200 µg/ml) or IgG2a isotype control (100 or 200 µg/ml). The mAT1R Ab clone (5.2a) was applied in two variants, either generated by hybridoma technique or recombinantly expressed. The specificity of AT1R Ab was tested by using the AT1R antagonist telmisartan (TEL, Germany). The response of the monocytes was measured via CCL18 secretion by commercial ELISA (see above).

Label-free dynamic mass redistribution (DMR) assay

Dynamic mass redistribution (DMR) measurements were conducted using the Epic biosensor system (Corning, USA) following the previously published protocols.[21,22] Briefly, 2.8 x 10^6 HEK293 cells were transfected in suspension with 1,500 ng expression plasmid encoding for the human AT1R using polyethylenimin (PEI, 1 mg/ml). Empty vector was used to adjust the total amount of DNA to 5,000 ng per transfection in a 10 cm dish. The following day, 18,000 cells per well were transferred to a 384-well Epic biosensor plate and incubated overnight at 37 °C. Then, cells were washed twice with Hanks' balanced salt solution (HBSS) (Thermo Fisher Scientific, USA) containing 20 mM HEPES and subsequently incubated in the DMR reader for 1 h at 37 °C to achieve temperature equilibration. When cells were pretreated with losartan, an AT1R antagonist, the latter was included in the equilibration period. After baseline recording, stimuli were added to the biosensor plate and alterations of DMR signals were monitored in real time.

Isolation and culture of primary dermal Fibroblasts (FBs)

FBs, from healthy donors have been generously provided by the Dermatology Department of the University Clinic Lübeck. Routine control for immunophenotypic marker fibrollin-1 was conducted. Each individual donor submitted an informed written consent and the ethics committee of the Medical Faculty of the University of Lübeck approved the study (21-191). FBs were cultured in RPMI-1640 high glucose, 100 IU/ml penicillin, 100 mg/ml streptomycin (all ThermoFisher Scientific, Karlsruhe, Germany) and 10% (v/v) Panexin (PAN Biotech, Aidenbach, Germany). All FBs used in experiments were between passages 3-6.

Stimulation of FBs, SDS-PAGE and Western blot analysis

FBs were stimulated for 5 days with 75% (v/v) RPMI-1640 high glucose, 100 IU/ml penicillin, 100 mg/ml streptomycin (all ThermoFisher Scientific, Karlsruhe, Germany), 10% (v/v) Panexin (PAN Biotech, Aidenbach, Germany) and 25% (v/v) pooled supernatant (SN) of monoclonal recombinant anti-AT1R antibody 5.2a- stimulated peripheral blood monocytes of healthy donors (n=10). As control, SN from isotype-control-stimulated monocytes were used. In addition, FBs were stimulated with the recombinant monoclonal anti-AT1R antibody 5.2.a (100 µg/ml).

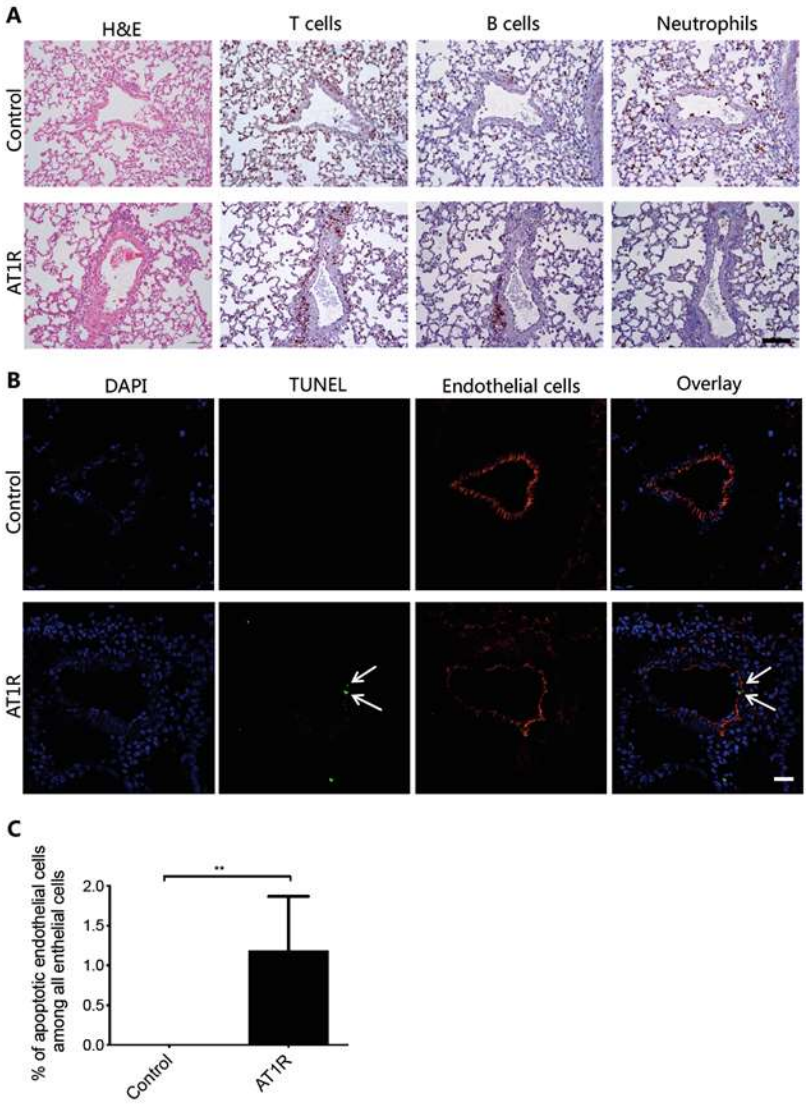
For SDS-PAGE 1 x 10^6 FBs were stimulated as indicated and lysed with RIPA buffer containing 1% SDS, the lysates were further incubated 4:1 with sample buffer (BioRad, Hercules, USA) containing 10% β-mercaptoethanol. Samples were analysed by semi-dry immunoblotting on methanol-activated PVDF membrane (BioRad, Munich, Germany). For immunodetection the following antibodies were used: alpha-smooth muscle actin (clone 1A4), CTGF (clone E2W5M), β-Actin-HRP conjugated (all Cell Signalling Technology, Danvers, MA, USA), collagen I (clone EPR7785, Abcam, Cambridge, GB) and goat anti rabbit IgG HRP. Densitometric analysis of Western blots was performed by applying Image Studio Life, relative to ß-actin.

Statistical analysis

All data are expressed as the mean ± SD. Statistical analysis was performed with a two-tailed paired and unpaired Student's t-test or one-way ANOVA, Bonferroni`s multiple comparison test was used for post-hoc test. For values that did not follow a Gaussian distribution, a two-tailed Mann-Whitney *U* test was applied. Fisher`s exact test was used to assess the significance of qualitative data. $P < 0.05$ was considered statistically significant.

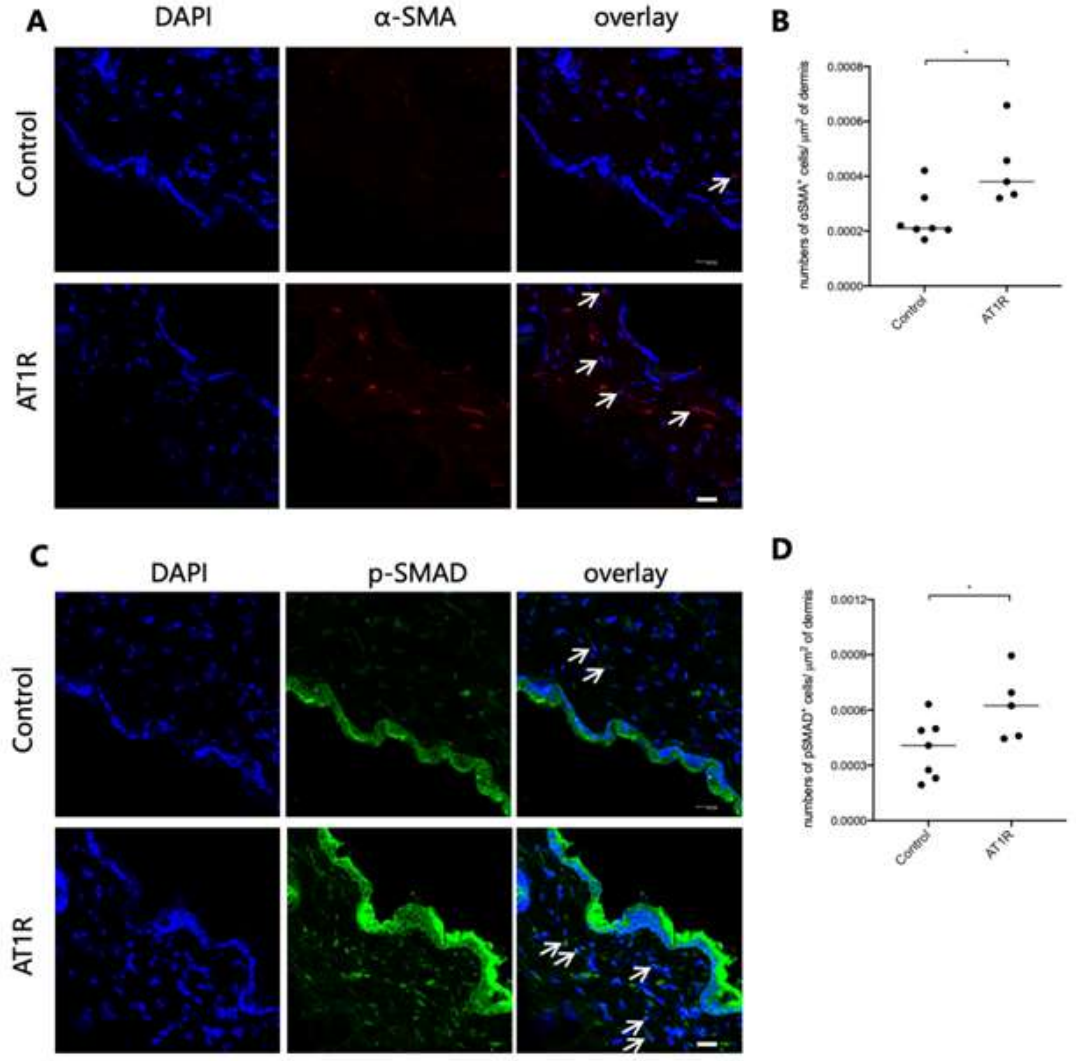
Supplemental file 2

supplementary figures



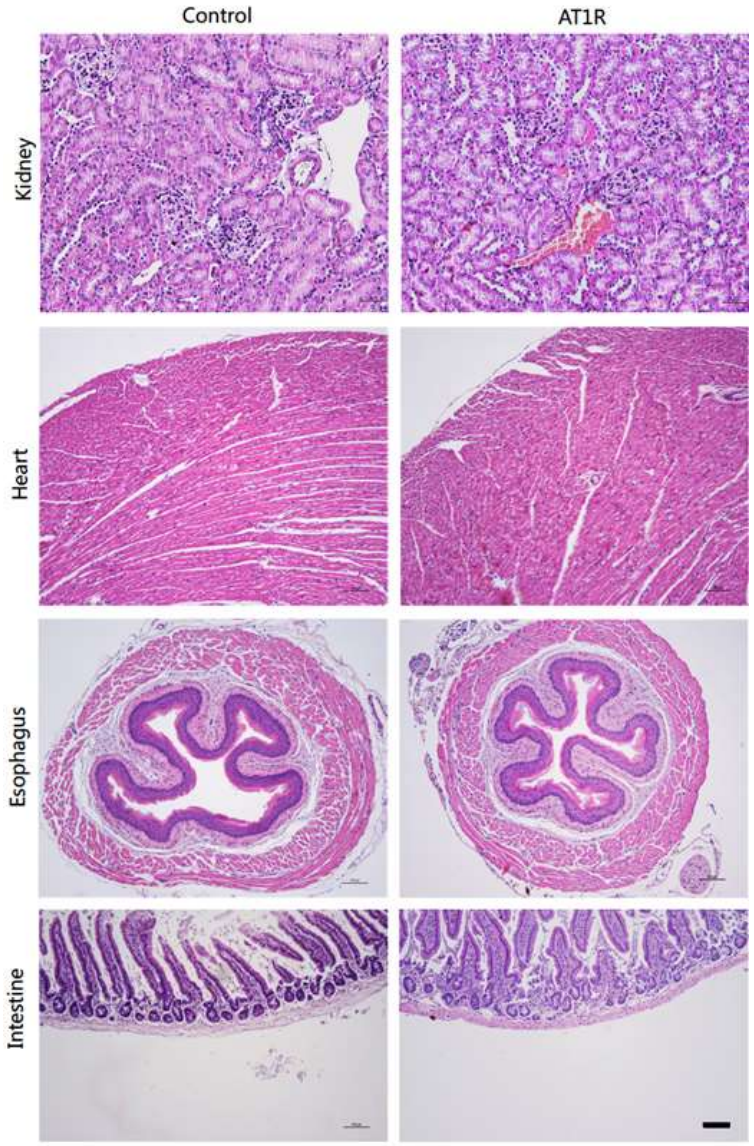
Supplementary Figure 1. Immunization with hAT1R induces perivascular inflammation and endothelial cell apoptosis in the lung. A: Perivascular inflammatory infiltrates in the lungs of AT1R-immunized mice. Representative micrographs of the histology and cellular composition of

the infiltrates in the lungs are shown (200x, scale bar=100μm). Histology was evaluated after H&E staining. Infiltrated T cells, B cells, and neutrophils were detected by immunohistochemical staining with anti-CD3, anti-CD45R and anti-neutrophil antibodies, respectively. B: Apoptotic endothelial cells in the lungs of hAT1R-immunized mice (630x, scale bar=25μm). Cryosections of lung tissue from hAT1R-immunized mice or control mice were incubated with a rat anti-mouse CD31 antibody and an Alexa Fluor 594-conjugated goat anti-rat antibody to detect endothelial cells (red), nucleus was stained with DAPI (blue). A TUNEL assay was performed to detect apoptotic cells (green). Representative micrographs of immunofluorescence staining are shown, and the white arrows indicate apoptotic endothelial cells. C: Quantitative analyses of apoptotic endothelial cells. For each lung sample, the percentage of apoptotic endothelial cells among all endothelial cells was calculated. The results are presented as the mean±SD, and the statistical analysis was performed using Student's t-test (** = $p<0.01$).

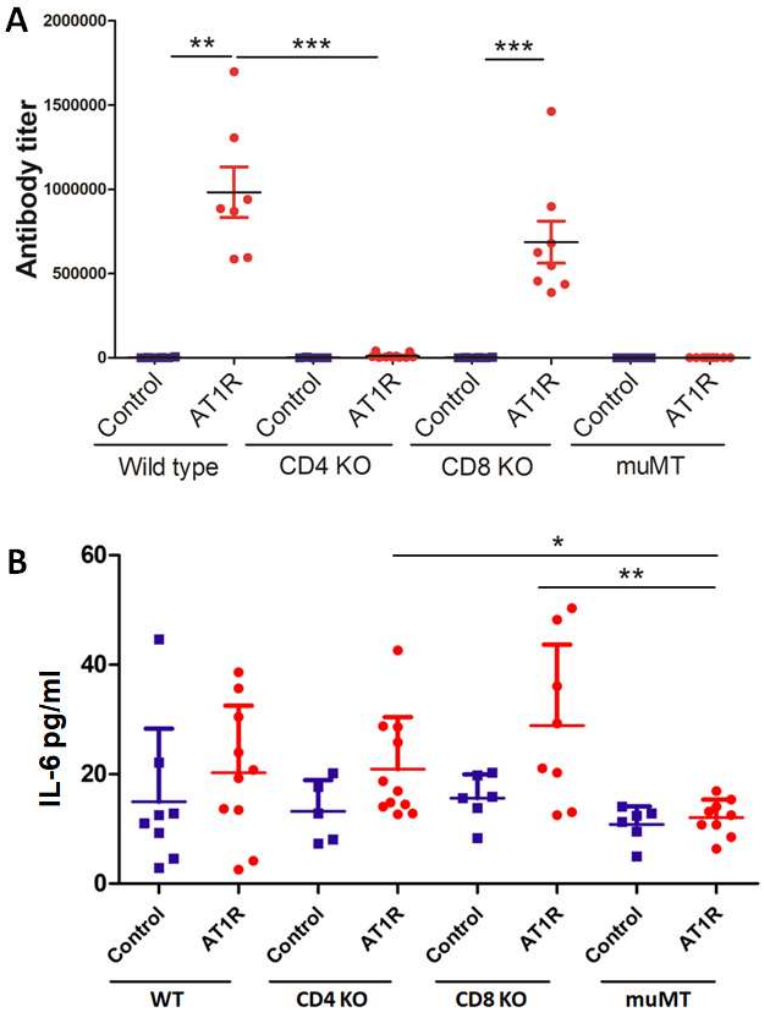


Supplementary Figure 2. Myofibroblast and activation of Smad signaling in murine skin. Numbers of myofibroblasts and p-smad2/3 positive cells were determined in the skin of hAT1R-immunized and control mice using immunofluorescence staining with anti-α-SMA (A) and anti-p-Smad2/3 (C) antibodies, respectively. Cryosections of skin tissue from hAT1R-immunized mice or control mice were incubated with a mouse anti-mouse α-SMA antibody and a Dylight 649-conjugated goat anti-mouse antibody to detect α-SMA positive cells (red), a goat anti mouse p-

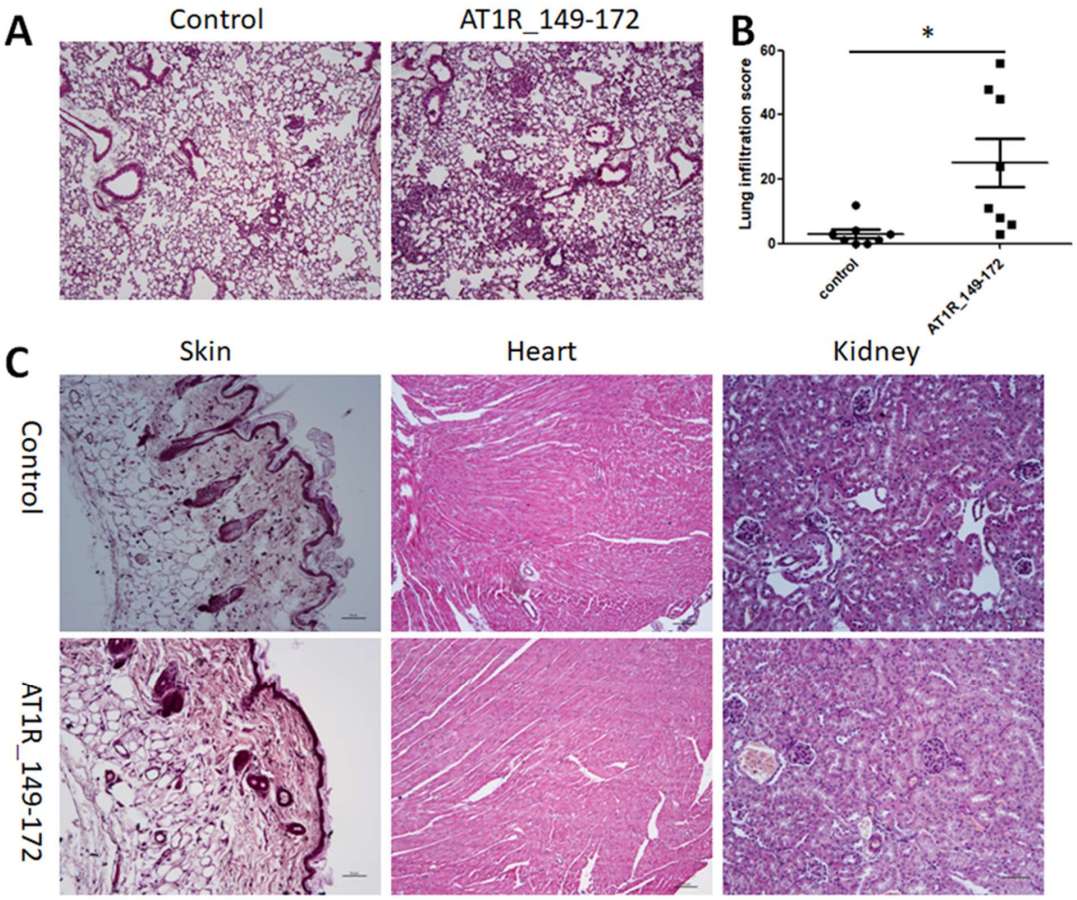
Smad2/3 antibody and Alexa 488-conjugated donkey anti-goat IgG antibody was applied to detect p-Smad2/3 positive cells (green), nuclei were stained with DAPI (blue). Representative micrographs of immunofluorescence staining are shown, and the white arrows indicate the positive cells. (630x, scale bar=25μm). B and D: Quantitative analyses of α-SMA positive cells and p-Smad2/3 positive cells. For each skin sample, the percentage of α-SMA positive cells and p-Smad2/3 positive cells to the area (μm^2) of skin examined was calculated. The results are presented as mean ± SD, and the statistical analysis was performed using Student's t-test (* = $p<0.05$).



Supplementary Figure 3. Immunization with hAT1R does not cause inflammation in the heart or gut. Internal organs were collected, and histological sections were evaluated by H&E staining. Representative micrographs are shown (100x, scale bar=100μm).



Supplementary Figure 4. Serum levels of anti-AT1R IgG (A) and IL-6 (B) in mice. Levels of anti-AT1R IgG and IL-6 were detected in sera of control ME-immunized or AT1R ME-immunized wild type (WT) , $CD4^+$ T cell deficient (CD4 KO), $CD8^+$ T cell deficient (CD8 KO) and B cell deficient (B KO, muMT) mice. P value was calculated by Mann Whitney test or Student t test. **, $p<0.01$, ***, $p<0.001$.



Supplementary Figure 5. Immunization with AT1R_149-172 peptide induced lung inflammation in mice. AT1R_149-172 CIIIWLLAGLASLPAIIHRNVFFI was predicted as the peptide showing the strongest binding affinity to murine MHC class II molecule H-2-IAb using NetMHCIIpan 3.1 software. Female C57BL/6J mice were immunized with 0.2 mg of AT1R_149-172 peptide or control solvent emulsified with an equal volume of complete Freund adjuvant by subcutaneous injection into the footpad. Two weeks after the first immunization, mice were boosted with the same amount of antigen emulsified with incomplete Freund adjuvant (IFA). Three weeks after the first immunization, mice were sacrificed and tissue samples including the lung, skin, kidney and heart were collected for histological evaluation using H&E staining. A.

Representative micrographs of histology of the lungs of and AT1R_149-172-immunized mice (n=8´) and control mice (n=8). B. Analysis of lung inflammation quantified by scoring the number of immune infiltrates in intra-alveolar areas in a double-blinded fashion. The results are presented as mean±SD. Statistical analysis was performed using Mann-Whitney test (* = $p<0.05$). C. Representative micrographs of histology of skin, heart and kidney of AT1R_149-172-immunized mice and control mice. No evidence of inflammation was observed in these organs.

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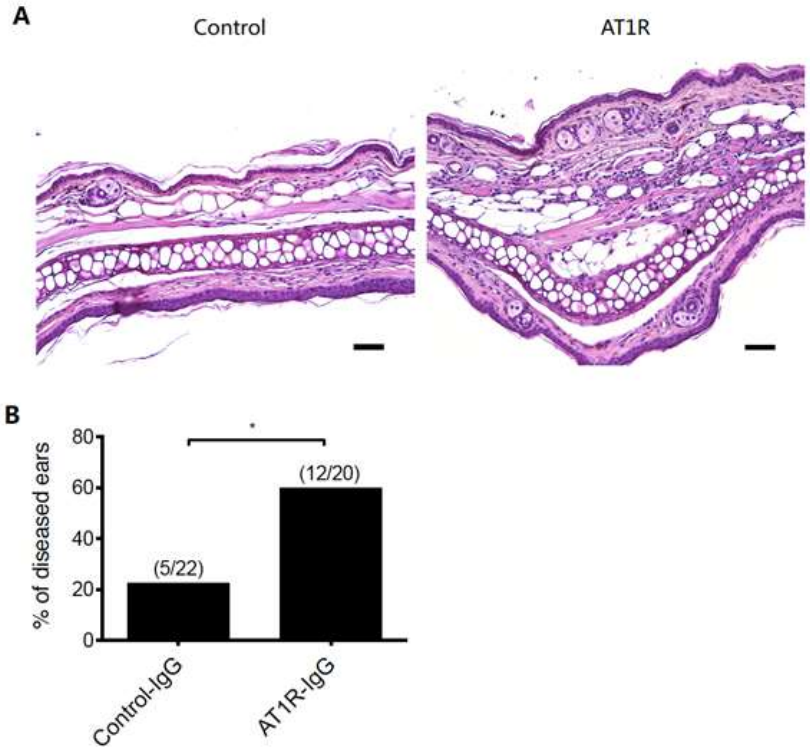
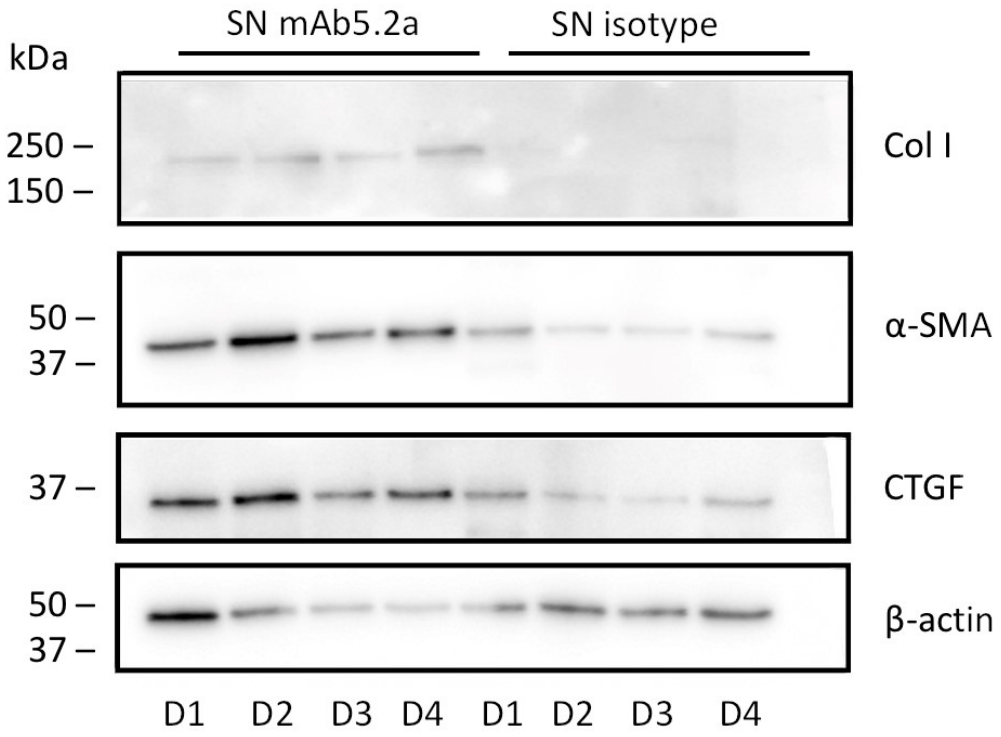


Supplementary Figure 6. Transfer of IgG isolated from hAT1R-immunized mice induces skin inflammation. A: Histology of ears injected with IgG isolated from control- (Control-IgG, left) or hAT1R-immunized mice (AT1R-IgG, right) (200x, scale bar=50μm). B: Incidence of inflammation in ears treated with control-IgG or AT1R-IgG. The numbers on top of the bars indicate the ratio between the number of diseased ears and the total number of treated ears. Statistical analyses were performed using Fisher's exact test (*= $p<0.05$).



Supplementary Figure 7: **Representative Western Blot analyses for the induction of fibrosis.** FBs from four different donors (D1-D4) were independently incubated with supernatant of monocytes stimulated with recombinant mAb 5.2a, isotype Ig2a control (shown) or recombinant mAb5.2a as control, lysed and subjected to western blot analysis after five days. For immune detection following antibodies against pro-fibrotic markers were used: Collagen I (Col I, ~ 220 kDa), alpha smooth muscle protein (α-SMA, ~ 42 kDa), connective tissue growth factor (CTGF, ~ 35 kDa). β-actin was used as loading control of cell lysates.