Online Supplemental Methods and Data

AAV Delivery of TNF-α shRNA Attenuates Cold-induced Pulmonary Hypertension and Pulmonary Arterial Remodeling

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Online Supplemental Methods

Effects of cold exposure on pulmonary TNF-α expression, macrophage infiltration and PA blood pressure. Twenty-four rats were exposed to moderate cold (5±0.5°C) while 8 rats were kept at room temperature (warm, 23.5 ± 0.5°C). At 3, 5, 7 and 14 days after exposure to cold, 6 cold-exposed rats and 2 warm-adapted rats were anesthetized with ketamine/xylazine (85/15 mg/kg IP) for measuring RV pressure (see below). Following RV pressure measurement, rats were euthanized and perfused with saline. Lungs and PAs were collected for western blot analysis. A part of these tissues was processed for paraffin embedding for IHC analysis.

Generation of recombinant AAV.TNF. Adeno-associated virus-2 vector (AAV2, Stratagene, La Jolla, CA USA) with TNFshRNA was constructed similar to previously described.[1-3] Briefly, the pAAV.TNFshRNA constructs were transfected into isolated pulmonary artery smooth muscle cells and inhibition efficiency was determined via Western blot analysis using an anti-TNF-α primary antibody (1μg/μl, Abcam Inc. Cambridge, MA, USA) as we previously described. AAV-carrying Scrambled shRNA (AAV.ScrshRNA) was and used as a control construct and was purchased from and confirmed by BD Biosciences (Palo Alto, CA. USA) not to match any known gene sequence.[1] AAV carrying GFP (AAV.GFP) was also amplified and injected into two animals for additional controls (Fig. S4). AAV.TNFshRNA, AAV.ScrshRNA, and AAV.GFP were then packaged with pHelper and pAAV-RC in modified AAV 293 cells to produce recombinant virus particles.[2-3] The titer was determined by real-time PCR.[4]

Animal Study protocols. This study was carried out according to the guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals. This project was approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (IACUC). Six groups of male Sprague–Dawley rats (145–180g, 8 rats/group) were allowed to acclimate for one week and then anesthetized with ketamine/xylazine (85/15 mg/kg, IP) and the left jugular vein was exposed for injection. Three groups of rats were injected with either AAV.TNFshRNA, AAV.ScrshRNA or PBS (300 μl), allowed to recover for 24 hours, and moved to a climate controlled walk-in cold chamber (5.0±0.5°C). The viral particles were delivered IV at $1.2x10^8$ PFU/300 μl/rat. The remaining three groups received identical injections but were kept in a climate chamber maintained 23.5±0.5°C (room temperature). After eight weeks of exposure to cold, the animals' right ventricular blood pressure was determined prior to euthanasia via overdose of ketamine/xylazine (200/20 mg/kg, IP). Two additional animals were injected with AAV.GFP and kept in warm conditions for determining AAV localization.

Measurement of right ventricular blood pressure. Eight weeks after injection, animals were anesthetized with ketamine/xylazine (85/15 mg/kg IP) and the right jugular vein was exposed. A blood pressure recording device (PA-C40, DSI Inc. St. Paul, MN, USA) was inserted into the jugular vein and maneuvered into the right ventricle for monitoring blood pressure. After a stable signal was established, the right ventricular (RV) blood pressure was recorded once per minute for a minimum of 15 minutes using DSI Dataquest A.R.T software (DSI Inc). This RV pressure is a reliable indicator of pulmonary arterial blood pressure and has been used by numerous investigators for evaluation of pulmonary pressure.[5-9] After recording the pressure, animals were euthanized immediately *via* overdose of ketamine/xylazine (200/20 mg/kg, IP) followed by exsanguination and purfused with heparin saline.

Morphometric analysis of small pulmonary arteries. Lung tissue was post-fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm thickness. Small and resistance PAs (50-80 μm in diameter) were examined. Basically, the morphometric measurements were taken approximately at the beginning of the 3rd order branch of PAs. At this position, the diameters small PAs were very close in the same group of animals. A total of 2-5 small PAs were examined randomly for each section in a series of 5 sections. The value was the average of 5-10 readings for each animal.

Right ventricular hypertrophy index. The right ventricle from each animal was dissected from the left ventricle (and septum), weighed and the hypertrophy index was calculated by normalizing the right ventricle weight to the left ventricle and septum weight.

Western blot analysis of TNF-α, IL-6, and PDE-1C protein expression in tissue. Pulmonary arteries and lungs were collected for Western blot analysis of protein expression of TNF-α (1μg/μl, Abcam, Cambridge, MA, USA), IL-6 (1:1000, Abcam) and PDE-1C (1:500, Santa Cruz Inc., Santa Cruz, CA, USA) as we described previously.[1, 10]

Immunohistochemical (IHC) analysis of macrophage infiltration. The IHC analysis was performed as described previously.[1, 10] Briefly, macrophage infiltration was assessed using a anti-CD-68 marker (1:100, Abcam). Both density of the CD-68 staining and the number of CD-68 positive cells were measured.

Isolation of pulmonary artery smooth muscle cells (PASMCs). PASMCs were isolated using the method adapted from Ray et al, 2002.[11] Briefly, PAs were placed in Fungizone solution (10 μl of 0.25 mg/ml Fungizone (Life Technologies, Carlsbad, CA, USA) in 10 ml Dulbecco's Modified Eagle Medium (DMEM) (catalog #12430-054, Life Technologies). The adventitia was removed and the artery was placed in fresh DMEM and cut into small segments. The artery segments were then subjected to enzyme digestion (5.5 ml DMEM with 13.9 mg collagenase type II, 10.9 mg elastase, and 5 mg soybean trypsin inhibitor, Worthington Biochem, Lakewood, NJ, USA) for 1-1.5 hrs at 37°C. After digestion, the solution was filtered using a 70 μm filter, centrifuged in fresh medium, and transferred to 12-well plates. The media was changed after 24 hrs (and every 48 hrs thereafter) and the cells were allowed to grow unmolested for 5-7 days, depending on confluence. After the cells grew to confluence, they were trypsinized, counted and seeded into 6 well plates ($3x10^4$ per well) for the PCNA and cGMP.

Measurement of vascular superoxide production in PASMCs. We measured superoxide production using the oxidation sensitive dye dihydroethidium (DHE, Sigma-Aldrich, Atlanta, GA, USA) in isolated PASMCs in 6 well plates. This method was provided in our previous studies.[12-14,15] DHE enters the cells and is oxidized by O_2^- to yield ethidium bromide (EB) which binds to DNA to produce bright red fluorescence. EB emits red fluorescence (610 nm) when excited at 488 nm.

Determination of cGMP in PASMCs. The cGMP level was determined using a cGMP-specific ELISA assay kit (KGE003, R&D Systems, Minneapolis, MN, USA) and a microplate reader (BioTek Inc.). Lysates of PASMCs were used for measuring cGMP according to the manufacturer's directions.

Semi-quantitative analysis of lung TNFα mRNA. TNFα mRNA was measured using RT-PCR as we described previously.[10]

Measurement of plasma TNFα. Plasma TNFα was measured using an ELISA kit according to the manufacturer's instruction (R&D).

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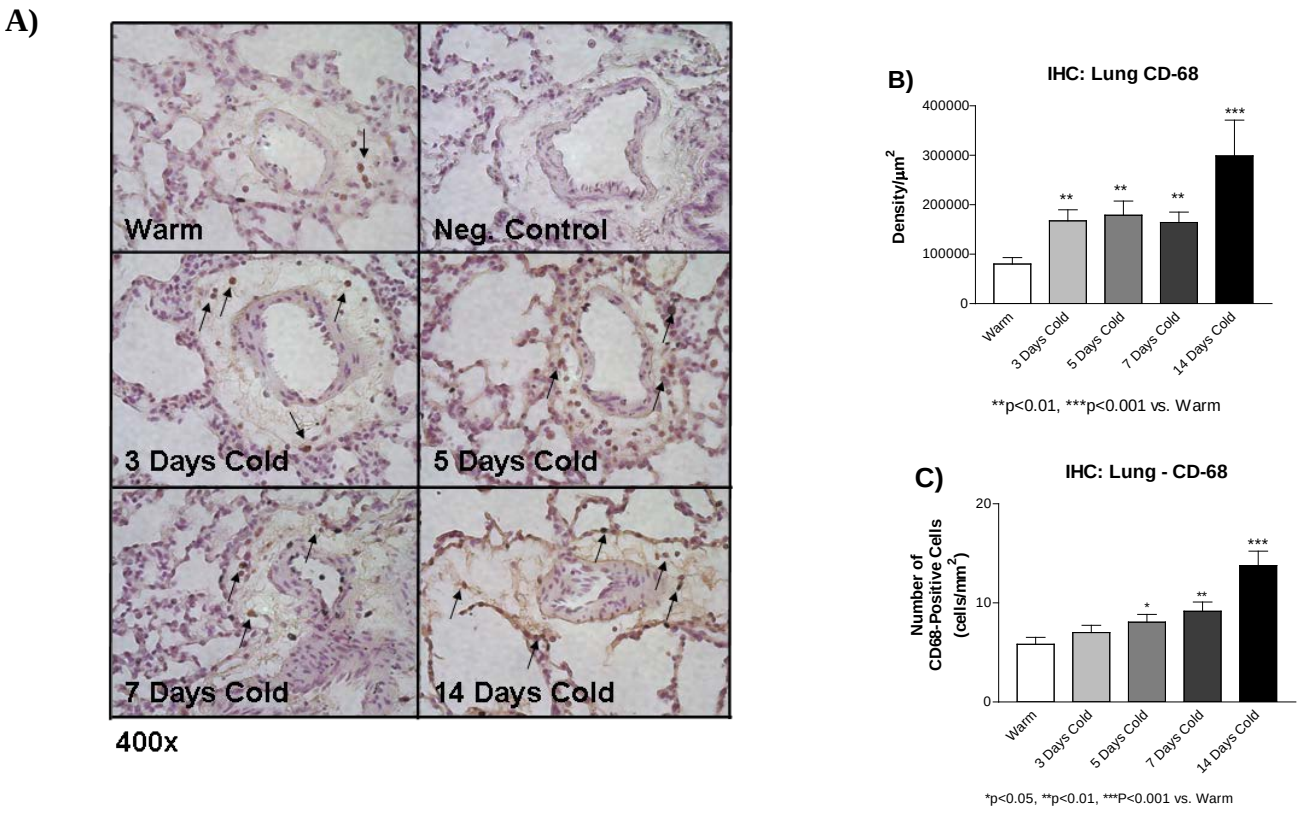
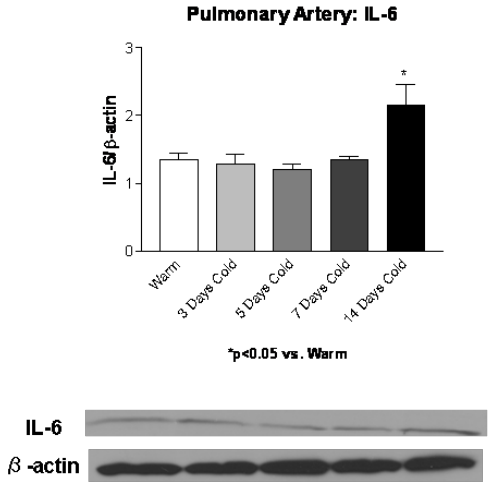
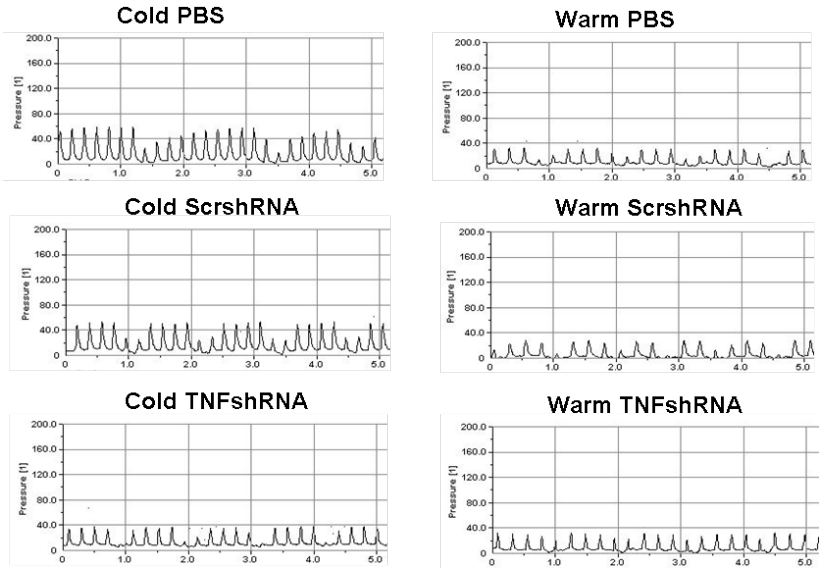
Online Supplemental Data



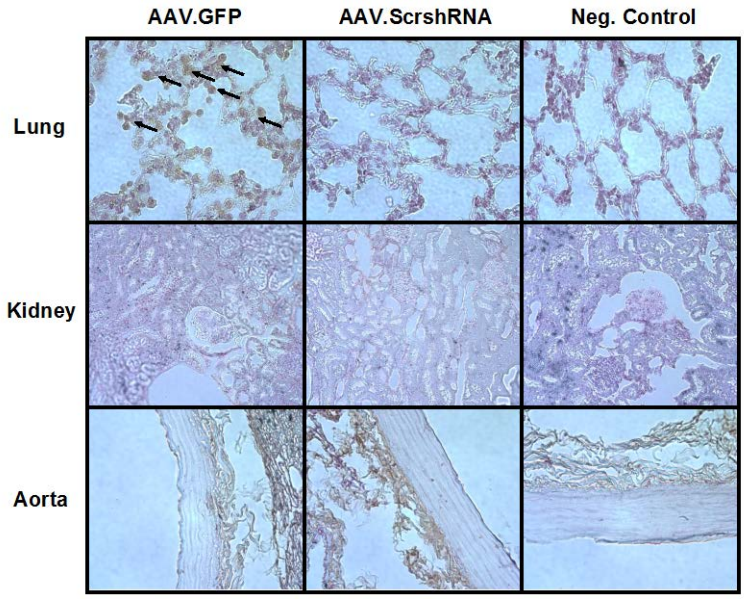
Supplemental Figure S1. Cold exposure increased macrophage infiltration in the lungs and around PAs. Macrophages were revealed using an anti-CD-68 marker. Macrophage infiltration was increased as early as 3 days after exposure to cold. **(A)** Photomicrographs of macrophage infiltration in the lungs. Arrows point to macrophages (dark brown staining). (B) Quantification of macrophage staining density. (C) Number of macrophage in the lungs. The data for this figure was obtained from rats exposed to cold for various lengths of time (3, 5, 7 or 14 days) while one group was kept in warm conditions (room temperature) as control. N=6.



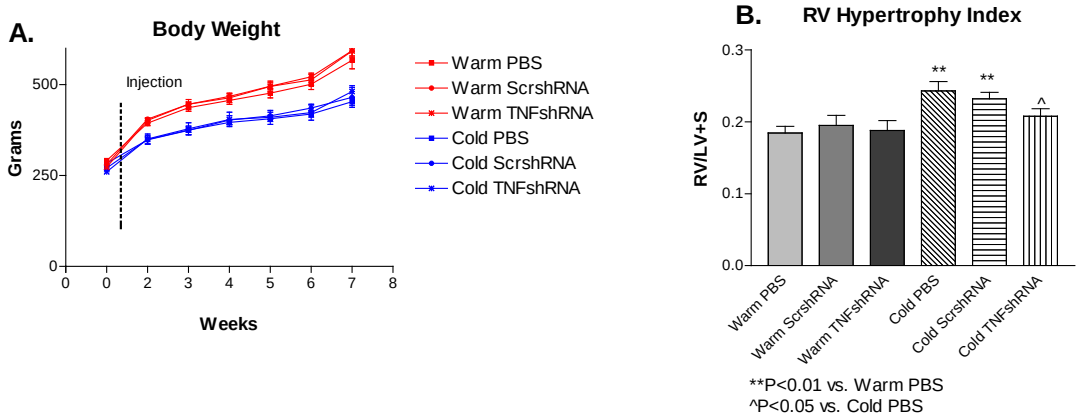
Supplemental Figure S2. Cold exposure did not increase PA IL-6 expression until 14 days after exposure to cold. Western blot analysis of IL-6 expression in PAs. Data=means±SEM. *p<0.05 *vs* the Warm group. N=6.



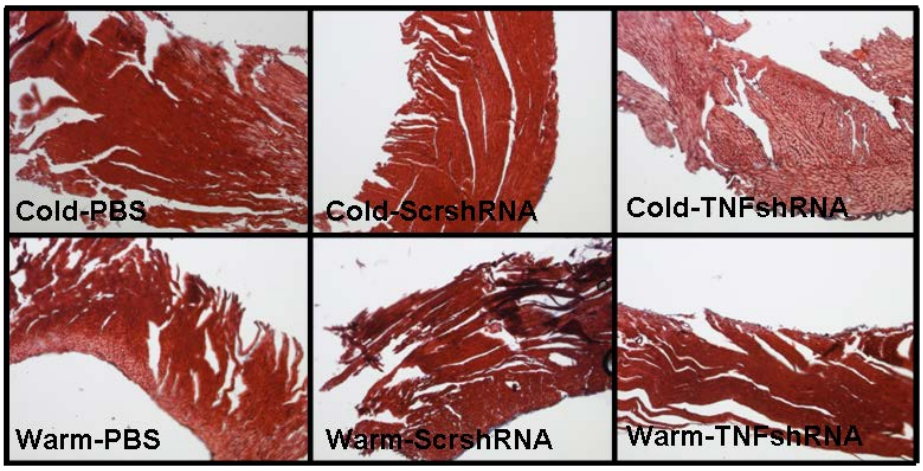
Supplemental Figure S3. TNFshRNA attenuated the cold-induced increase in right ventricle (RV) systolic blood pressure. RV blood pressure was recorded 8 weeks after treatment with TNFshRNA using a telemetry system. Refer to Figure 2 for quantitative analysis of RV systolic blood pressure. N=6.



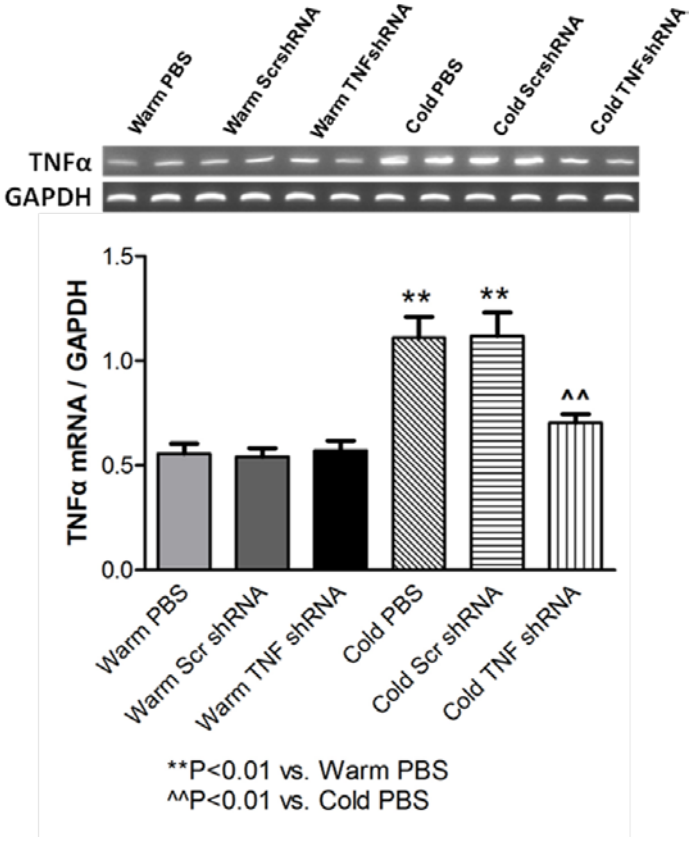
Supplemental Figure S4. AAV.GFP was expressed in the lung. Two animals were injected, *via* jugular veins, with the AAV.GFP construct in a similar manner to the AAV.TNFshRNA- and AAV.ScrshRNA-treated animals. After eight weeks, these animals were euthanized and perfused with paraformaldehyde. Lungs, kidneys, and aortas were psot-fixed, paraffin embedded, and sectioned at 5 μm. The tissue sections were stained with an anti-GFP specific antibody (Abcam, 1:500) followed by a secondary antibody conjugated to horseradish peroxidase (HRP, Abcam, 1:2000) to evaluate GFP expression. The AAV.ScrshRNA and PBS Control lung sections showed no positive GFP expression. Lung sections from AAV.GFP animals, however, showed strong GFP staining (brown color, indicated by arrows) throughout the lung samples. Therefore, the GFP viral construct successfully integrated into the lung tissue and produced GFP protein, indicating the successful construction of AVV constructs. In contrast, kidney and aorta sections did not show any GFP expression in the AAV.GFP-treated animals. Therefore, GFP was expressed in the lung but not systemically (kidneys, aorta), suggesting that the viral particles may be primarily trapped in the pulmonary circulation and lungs following delivery *via* jugular veins. The AAV-mediated gene expression still exists at 8 weeks after gene delivery (length of the study).



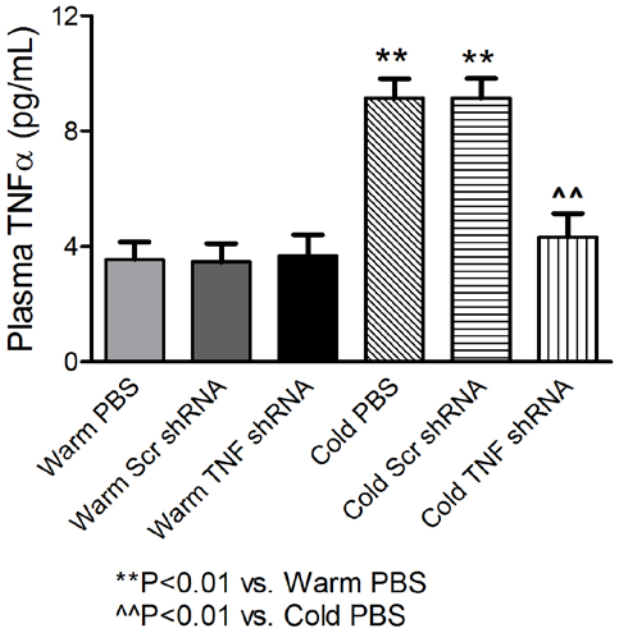
Supplemental Figure S5. Body weight and right ventricle (RV) weight. A) TNFshRNA did not affect body weight gain. Body weight was decreased in all cold groups, a phenomenon seen in cold-exposed animals. However, no significant difference was found between groups in either cold or warm environments, indicating that viral delivery did not affect the body weight gain. **B)** Cold exposure increased the RV weight significantly (RV hypertrophy). TNFshRNA prevented cold-induced RV hypertrophy (RVH). The right ventricle was dissected from the left ventricle (and septum) to determine the hypertrophic index. Data=means±SEM. **p<0.01 vs. Warm PBS; ^p<0.05 vs. Cold PBS. N=6.



Supplemental Figure S6. Trichrome staining indicated that collagen deposition was not detectable in the RV of rats kept at either temperature environment.



Supplemental Figure S7. TNFshRNA abolished the cold-induced increase in TNFα mRNA expression in lungs. TNFα mRNA was measured using RT-PCR. The bar graph showed semi-quantitative analysis of TNFα mRNA. Data=means±SEM. **p<0.01 vs. Warm PBS; ^^p<0.001 *vs.* Cold PBS. N=6.



Supplemental Figure S8. TNFshRNA abolished the cold-induced increase in plasma TNFα. Plasma TNFα was measured using an ELISA kit (R&D). Data=means±SEM. **p<0.01 vs. Warm PBS; ^^p<0.001 vs. Cold PBS. N=6.