Online Data Supplement

RNAi Inhibition of Interleukin-6 Attenuates Cold-Induced Hypertension

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Supplemental Methods

AAV.IL-6 generation. Three sets of complimentary rat IL-6 siRNA oligos flanked by BamHI and EcoRI restriction sites were designed using Gene Link RNAi Explorer software (Accesion No: NM_012589) and synthesized by IDT DNA (Coralvill, IA, USA). The complimentary oligos were then annealed and ligated into pAAV-U6shRNA (Adeno-associated virus-2 vector, previously published) vector forming pAAV.IL-6shRNA. The fidelity was measured by sequencing of the vector/insert sequence using the upstream primer U6-p39 5'-GGT CCT AAG GTA GCG AAA GC-'3 and compared the sequence using Blast. The pAAV.IL-6shRNA constructs were then transfected into 3T3-L1 adipocytes (ATCC) and inhibition efficiency was determined via Western blot analysis using a rabbit polyclonal anti-IL-6 primary antibody (Abcam Inc.). One construct was selected and then transfected into and amplified by *E.coli.* The *E.coli* was then lysed and the plasmid DNA was removed and purified *via* CsCl gradient and ultracentrifugation.

Packaging of recombinant plasmids of adeno-associated virus with IL-6-shRNA. One IL-6shRNA that achieved the greatest inhibition was chosen for the *in vivo* study. Adeno-associated virus-2 vector (Stratagene, La Jolla, CA USA) with RNA polymerase III promoter U6 and IL-6-shRNA were constructed as previously described by our lab[1]. AAV.U6-IL-6shRNA was then packaged with pHelper and pAAV-RC to produce recombinant AAV.IL-6shRNA. The titer was determined by real-time PCR[1-2].

Additional AAV generation. AAV.ControlshRNA was purchased from and tested by BD Biosciences (Palo Alto, CA. USA) not to match any known gene sequence.

Animals. 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 Institutional Animal Care and Use Committee. We used 4 groups of male Sprague–Dawley rats (145–180g, 6 rats/group). All rats were housed individually in wire-mesh cages and were provided with Purina laboratory chow (No. 5001) and tap water ad libitum throughout the experiment.

Animal study protocols. The rats were allowed to acclimate for a week. After the acclimation period, resting systolic BP and body wirght were measured twice weekly at room temperature from the tail of each unanesthetized rat using the tail-cuff method with slight warming (28°C), but not heating of the tail. All rats were handled frequently (twice a day) to minimize handling stress. Animals did not appear stressed during the BP measurement. The tail-cuff measurements were performed at room temperature (25°C) between 8:00-12:00 am using a CODA 6 blood pressure monitor. The volume-based tail-cuff measurements of BP have been validated by using a telemetry system[3]. An average of 6 measurements was obtained for each session. After two steady state blood pressure readings were obtained, the rats were treated as described below:

Each group consisted of 6 rats.
Group 1. 500µl AAV.ScshRNA (1.2×10^8 PFU/rat via tail vein) (Sc=scrambled sequence)
Group 2. 500µl AAV.IL-6shRNA (1.2×10^8 PFU/rat via tail vein)
Group 3. 500µl PBS via tail vein

Group 4. 500µl PBS via tail vein

Following injections, three groups (AAV.IL-6shRNA, AAV.ScrambledshRNA and one PBS) were moved into to a climate-controlled walk-in chamber (5 ± 0.1°C), whereas the remaining PBS group was kept in an identical chamber maintained at RT (25 ± 0.1°C, warm). BP was measured 24 h after the injection to ensure that the gene delivery did not affect animal-resting BP at room temperature. BP and BW were measured at least once a week throughout the experiment. During week eight after viral injection, 3 animals from each group were deeply anesthetized with pentobarbital sodium (120 mg/kg i.p.) for blood collection, followed by transcardiac perfusion with heparinized phosphate buffered saline (PBS). The heart and kidneys were removed and weighed. The kidneys, heart and aorta were divided then OCT embedded for dihydroethidium (DHE) staining and for Western blot analysis of IL-6 and TNF-α protein expression. The remaining 3 animals from each group were then deeply anesthetized for blood collection, followed by transcardiac perfusion with PBS then perfused a second time with 4% paraformaldehyde (PFA) in PBS. The heart and kidneys were removed and weighed. The heart, kidneys, and aorta were fixed in 4% PFA in PBS overnight and paraffin embedded. 5µm sections were cut and slides were used for immunohistochemical (IHC) and hematoxylin and eosin stain (H&E) histological analyses.

Four groups were exposed to different temperature conditions as follows:
ScshRNA-Cold, rats treated with scrambled RNA and exposed to cold (5°C)
AAV.IL-6shRNA-Cold, rats treated with IL-6shRNA and exposed to cold (5°C)
PBS-Cold, rats treated with PBS and exposed to cold (5°C)
PBS-Warm, rats treated with PBS and kept at room temperature (warm, 25°C)

Western blot analysis of IL-6 and TNF-α protein expression in tissue. Tissues were frozen in liquid nitrogen, shattered, and powderized using a mortar and pestle. The frozen powder was then homogenized in lysis buffer (50 mM Tris, 150 mM NaCl, 1% nonidet p40, .5% sodium deoxycholate, .1% sodium dodecyl sulfate) containing a protease inhibitor complex, incubated on ice for 30 minutes and centrifuged for 10 min at 10,000 *g*. Supernatants were collected and immediately mixed with an equal volume of electrophoresis loading buffer for Western blot analysis of IL-6 and TNF-α proteins. TNF-α and IL-6 expression was normalized with the expression of β-actin, which served as an internal control. Briefly, tissue proteins were equalized after subjection to bicinchoninic acid (BCA) quantification. Equal protein concentrations were loaded for all groups. For IL-6, membranes were blocked for 2 hours at room temperature in 5% milk/TBS-T. Membranes were incubated with rabbit polyclonal anti-IL-6 primary antibody (dilution 1:1,000; Abcam Inc.) at 4°C overnight and then goat anti-rabbit secondary antibody (dilution 1:2,000 Santa Cruz Biotechnology) for 1 hour at room temperature. For TNF-α, membranes were blocked in 2% bovine serum albumin (BSA) for 2 hours at room temperature followed by incubation with rabbit polyclonal anti-TNF-α primary antibody (dilution 1:1,000 Cell Signaling) overnight in 4°. Membranes were then incubated in goat anti-rabbit secondary antibody for 1.5 hours room temperature. For β-actin, the membranes were blocked in 5% milk/TBS-T for 2 hours at room temperature followed by incubation with mouse monoclonal anti-β-actin primary antibody (dilution 1:10,000; Abcam Inc) for 1 hour room temperature and then with goat anti-mouse secondary antibody (dilution 1:2,000; Santa Cruz Biotechnology) for 1 hour room temperature. Enhanced chemiluminescence (Amersham) was added to the

membranes and exposed to photosensitive films. The films were imaged by using an X-ray processor (Konica Minolta, SRX-101A). Protein band intensities were quantified using Image J software.

IHC analysis of IL-6, CD-3 and CD-68 expression. After a 24-h fixation with 4% PFA, the heart, kidneys, and aorta were incubated overnight in 70% ethanol at 4°C and paraffin embedded. Sections (5 µm) were used for the immunostaining of IL-6, CD-3 and CD-68. For immunostaining, the sections were heated for 10 min with sodium citrate buffer (pH 6.0) for antigen retrieval, incubated for 15 min with 3% hydrogen peroxide solution (VWR International)**,** and 10 min with protein blocker (Background Sniper; Biocare Medical). For IL-6, the sections were incubated with rabbit polyclonal anti-IL-6 primary antibody (dilution 1:1,000; Abcam Inc) overnight and then with a goat anti-rabbit secondary antibody (dilution 1:500; Santa Cruz Biotechnology) for 1 hour. For CD-3, the sections were incubated with rabbit polyclonal anti-CD-3 primary antibody (dilution 1:1000; Abcam Inc) overnight and then with a goat anti-rabbit secondary antibody (dilution 1:2000; Santa Cruz Biotechnology) for 1 hour. For CD-68, the sections were incubated with mouse monoclonal anti-CD68 primary antibody (dilution 1:100; Abcam Inc) overnight and then with a goat anti-mouse secondary antibody (dilution 1:500; Santa Cruz Biotechnology) for 1 hour. The sections were then examined and photographed using a Nikon Eclipse Ti-U photomicroscope coupled with a digital color camera. The expression of IL-6, CD-3 and CD-68 expression level was evaluated by calculating the percentage of staining for a given area. In addition, counts of actual positively stained cells were performed for CD-3 and CD-68. To determine the antibody specificity, sections from each tissue were subjected to primary antibody only, secondary antibody only, and PBS treatment. These slides were considered negative controls and were compared to non-control slides.

Measurement of In Situ Superoxide Production.
We measured superoxide production by fluorescence microscopy using the oxidation sensitive dye dihydroethidium (DHE, Sigma-Aldrich, Atlanta, GA, USA). Using OCT embedded aorta tissue, sections were cut at 10 µm thickness. Excess OCT was removed from the slides using PBS. DHE (10µg/ml) was then allowed to incubate for 20 minutes at 37ºC in the dark. Excess DHE was then rinsed out using PBS. A nuclear stain was then applied using DAPI (Santa Cruz Biotechnology Inc, Santa Cruz, CA USA) and the slides were immediately viewed using a Nikon Eclipse Ti-U photomicroscope coupled with a digital color camera.

Statistical analysis. The data for BP and BW were analyzed by a repeated measures one-way analysis of variance (ANOVA). The protein expression and ROS levels were analyzed by one-way ANOVA. Turkey's multiple comparison test was used to assess the significance of differences between means. Significance was set at a 95% confidence limit.

References

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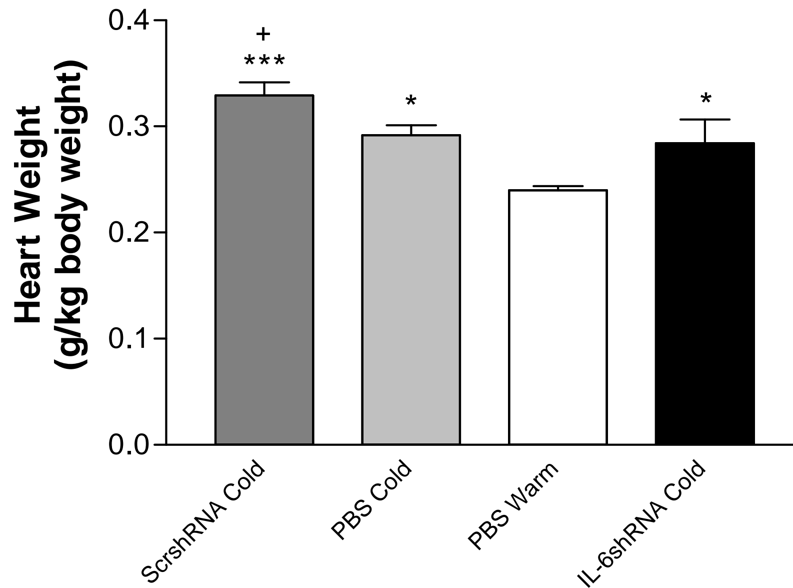
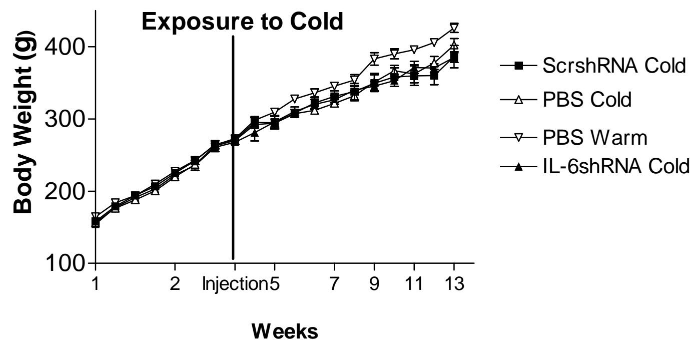
Supplemental Results



S1. Heart weight. Animals were euthanized at the end of week 8 of exposure to cold. Heart weight was adjusted for body weight (g/kg body weight) for each animal. Chronic exposure to cold increased the heart weight significantly. IL-6shRNA significantly attenuated the cold-induced increase in heart weight compared to the ScrshRNA Cold group. However, heart weight of the IL-6shRNA Cold group was significantly greater than that of the PBS Warm group, suggesting partial attenuation of cold-induced cardiac hypertrophy. *p<0.05, ***p<0.001 *vs* the PBS Warm group; +P<0.05 *vs* the IL-6shRNA Cold group. Data=means+SE. n=6.



S2. Body weight.