Supplement

Cellular sources of IL-6 and associations with clinical phenotypes and outcomes in PAH

Catherine E. Simpson[1*], Jenny Y. Chen[2*], Rachel L. Damico[1], Paul M. Hassoun[1], Lisa J. Martin[3], Jun Yang[4], Melanie Nies[4], Megan Griffiths[4], R. Dhananjay Vaidya[5], Stephanie Brandal[4], Michael W. Pauciulo[3], Katie A. Lutz[3], Anna W. Coleman[3], Eric D. Austin[6], Dunbar D. Ivy[7], William C. Nichols[3], and Allen D. Everett[4]

[1]Johns Hopkins University Department of Medicine, Division of Pulmonary and Critical Care Medicine
[2]Johns Hopkins University School of Medicine
[3]Division of Human Genetics, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine
[4]Johns Hopkins University Department of Pediatrics, Division of Pediatric Cardiology
[5]Johns Hopkins University Department of Medicine, Division of General Internal Medicine
[6]Vanderbilt University Department of Pediatrics, Division of Allergy, Immunology, and Pulmonary Medicine
[7]Children's Hospital Colorado, Department of Pediatric Cardiology
[*]These authors contributed equally to this article.

Corresponding Author:
Allen D. Everett
1800 Orleans St.
Room M2303
Baltimore, MD 21287
USA
Email: aeveret3@jhmi.edu

Supplemental Methods

Cell Lines and Cell Culture:

Primary pulmonary artery smooth muscle (PASMC) and endothelial cells (PAEC) were obtained from the Pulmonary Hypertension Breakthrough Initiative (PHBI) cell core facility (University of Pennsylvania) funded by the Cardiovascular Medical Research and Education Fund (CMREF). The cells were isolated from small pulmonary arteries of transplanted patients with severe PAH (N=22) or non-transplanted donor lungs (N=11). All cells were maintained at low passage (passage 3-8) under normal culture conditions in a humidified 5% CO_2-supplemented incubator at 37°C (Napco 8000 DH, ThermoFisher Scientific). Each cell type was cultured in a specific medium: PASMC in VascuLife SMC Medium (Cat# LL-0014, Lifeline Cell Technology, Frederick, MD) and PAEC in VascuLife VEGF-Mv Endothelial Medium (Cat# LL-0005, Lifeline Cell Technology, Frederick, MD). The cell conditioned media were harvested and centrifuged at 3000 rpm for 5 minutes at 4°C to remove cell debris then aliquoted and stored at -80°C.

RNA Extraction and RNAseq Analysis:

PASMCs and PAECs in normal culture conditions were subjected to RNA extraction after reaching 80-90% confluence. TRIzol reagent (Cat# 15596026, ThermoFisher Scientific) was used for total RNA extraction then the manufacturer's instructions (ArrayStar 6G RNAseq service, Rockville, MD) were followed to perform the RNAseq experiments. After RNAseq library preparation, libraries were sequenced for 150 cycles for both ends on an Illumina NovaSeq 6000 instrument. Image analysis and base calling were performed using the Solexa pipeline v1.8 (Off-Line Base Caller software, v1.8). Sequence quality was examined using the FastQC software [1]. The trimmed reads [2] were aligned to

reference genomes using Hisat2 software [3]. The transcript abundances for each sample were estimated with StringTie [4], and the fragments per kilobase of exon model per million reads mapped (FPKM) values for gene and transcript levels were calculated with R package Ballgown [5–7]. IL-6 gene expression levels (FPKM values) were extracted from the data analysis results. RNAseq raw data are currently being uploaded to Gene Expression Omnibus (GEO).

ELISA for IL-6:

Electrochemiluminescent sandwich immunosorbent assays for measuring IL-6 were performed using robotically spotted capture antibodies (Cat# D21AK-3, Meso Scale Discovery [MSD], Gaithersburg, MD) on an MSD 96-well plate. Capture antibody-spotted plates were blocked with 5% BSA-PBS complemented with .05% TWEEN (PBS-T) and incubated at room temperature on an orbital shaker (500 rpm) for 60 minutes. Calibrator for IL-6 (Cat# C0049-2, MSD) was used at a concentration range of 0.024-100 pg/mL via a 1:4 series dilution with diluent II (Cat# R51BB-3, MSD). Samples were diluted 15x in the same diluent II (Cat# R51BB-3, MSD) before being added onto the ELISA plate. The plates containing samples/calibrators were incubated at room temperature with shaking for 2 hours then washed with PBS-T 3 times. The IL-6 sulfo-tagged detection antibody cocktail (Cat# D21AK, 50X, MSD) was diluted in diluent III (Cat# R51BA-5, MSD) to 1X and added onto the plates. Finally, after one hour of incubation followed by PBS-T washing, 150 µl of 1X read buffer (Cat# R92TC-1, MSD) was added into each well, and the plate was promptly read in an MSD Sector Imager 2400. Inter-assay reliability as measured by percent coefficient of variation was 6.5 $\pm$ 3.2% (mean $\pm$ SD). All assays were performed in a single laboratory and by the same laboratory specialist.

Supplemental References

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5. Fu J, Frazee AC, Collado-Torres L, Jaffe, Andrew E. Leek JT. ballgown: Flexible, isoform-level differential expression analysis. 2016.
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Supplemental Tables and Figures

Table S1. Demographics and IL-6 Levels of Subjects with PAH versus Healthy Controls

	PAH	Controls	p value
Demographics			
Subjects, n	2017	60	
Age, years	55 (15)	35 (9)	<0.01
Sex, n female (%)	1611 (79.9)	47 (78.3)	0.45
Race, n Caucasian (%)	1662 (82.4)	49 (81.7)	0.91
IL-6 Levels			
IL-6, pg/mL (median, IQR)	1.82 (0.86-3.34)	0 (0-2.72)	<0.01

All data presented as mean (SD) unless otherwise specified. p values reflect comparisons between PAH cases and controls. See Table 1 for abbreviations.

Table S2. Demographics and IL-6 Levels of PAH Patients and Non-Transplanted Donors of PHBI Cell Lines

	SMC-PAH*	EC-PAH*	SMC-Control	EC-Control
Demographics				
Subjects, n	16	8	5	6
Age, years (median)	36	37	52	43
Sex, n female (%)	10 (62.5)	3 (37.5)	2 (40.0)	4 (66.7)
Race, n Caucasian (%)	12 (75.0)	7 (87.5)	5 (100.0)	6 (100.0)
PAH subtypes, n IPAH/APAH/FPAH	6/7/3	4/2/2		
IL-6 Levels				
IL-6, pg/mL (median, IQR)	12,301 (1694-21,822)	398 (298-525)	2554 (2253-14,799)	245 (116-400)

Definition of abbreviations: SMC: smooth muscle cell; EC: endothelial cell; APAH: disease-associated PAH. See Table 1 for abbreviations.
** Demographic information was missing for one PAH patient who provided both SMC-PAH and EC-PAH samples*

Table S3. PAH Subtypes for Subjects without Available Survival Data

Diagnosis	Number of Patients (overall n=33)
Disease Subtype	
CTD-PAH	18
IPAH	8
FPAH	2
Congenital heart disease	3
Portopulmonary hypertension	1
APAH, other	1

Definition of abbreviations: APAH: disease-associated PAH. See Table 1 for abbreviations.

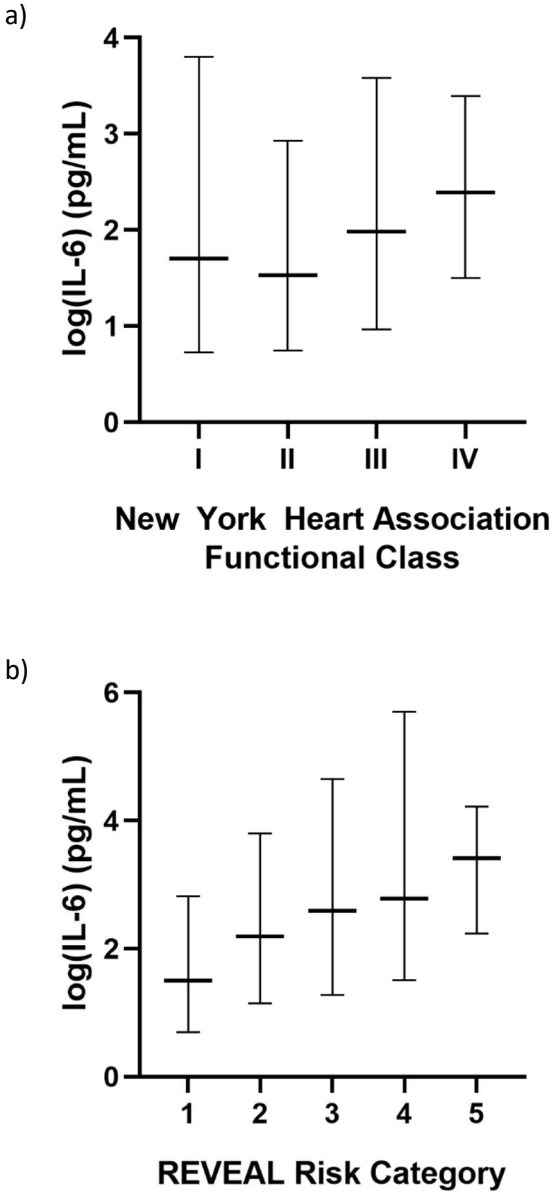

Figure S1. Comparison of interleukin 6 (IL-6) levels by: a) New York Heart Association Functional Class (NYHA FC), and b) REVEAL risk category ($p<0.001$). 25^{th} percentile, median, and 75^{th} percentile values are presented for each functional class and risk category.