SUPPLEMENTAL MATERIAL

Interleukin-6 mediates neutrophil mobilization from bone marrow in pulmonary hypertension

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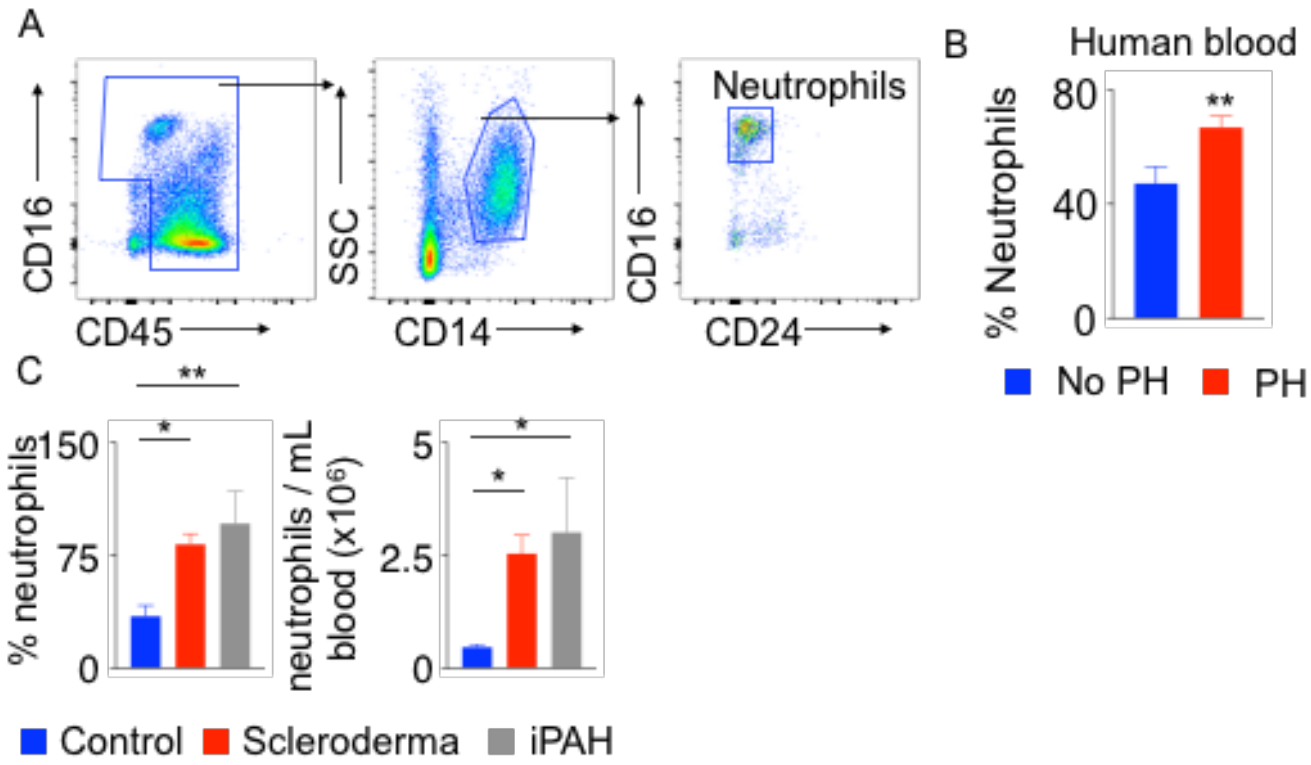
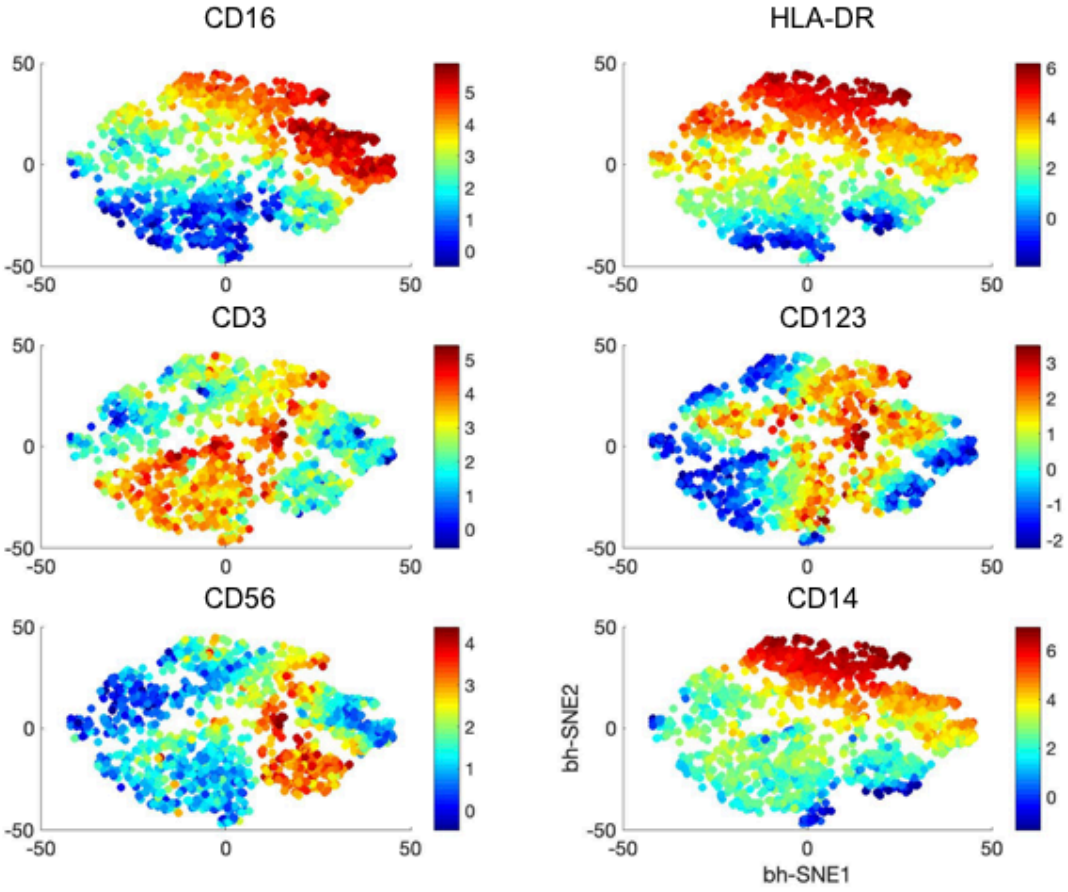
Supplemental Figure 1



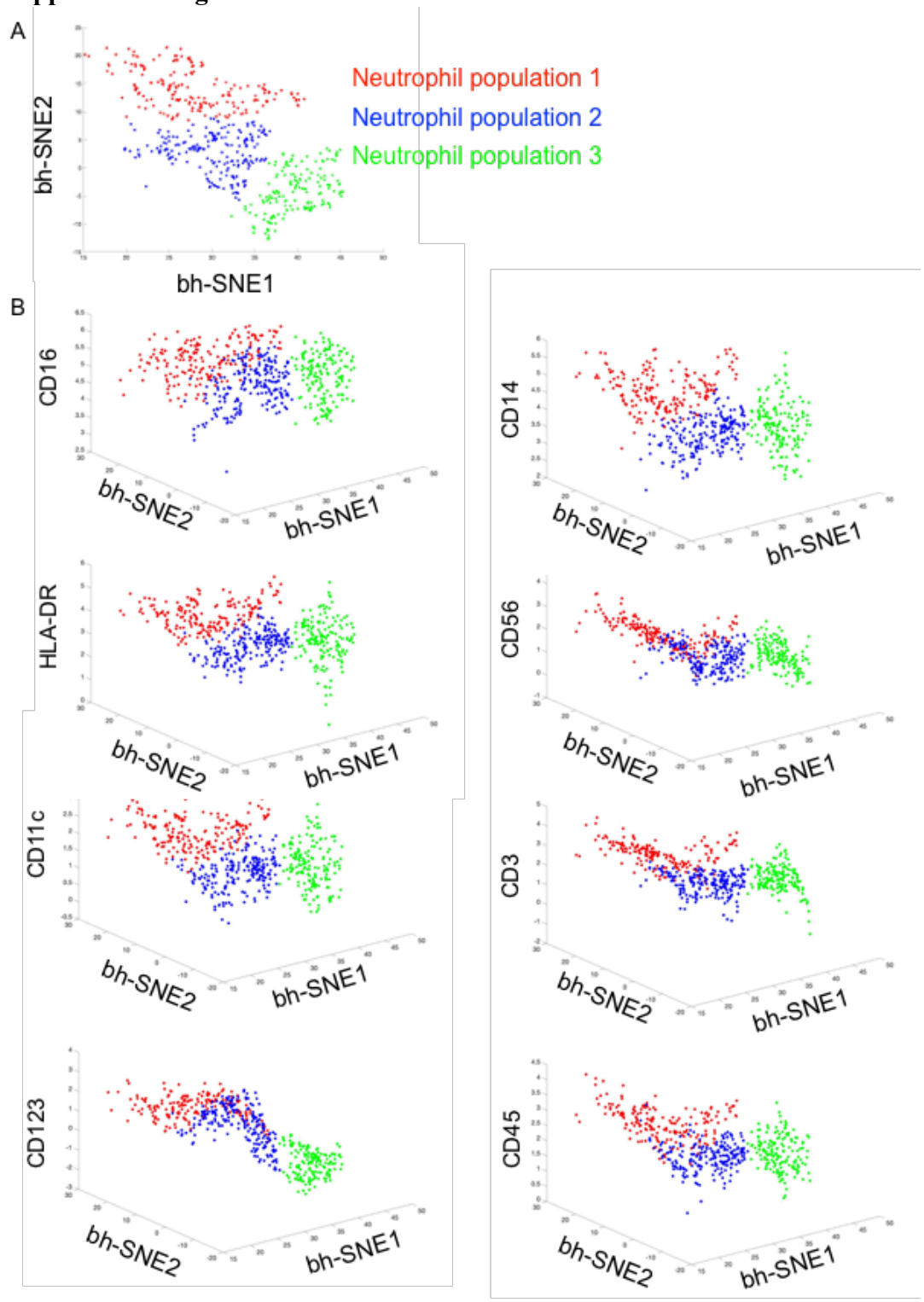
Supplemental Figure 1: The frequency of neutrophils in the lungs PH patients is increased. **A**) Flow plots showing the gating strategy of human blood neutrophils. B) The percentage of human neutrophils among myeloid cells was quantified in the blood. C) Neutrophil frequency and number were assessed by Flow Cytometry. n=5 samples per group. Mean ± s.e.m., * $P < 0.05$, ** $P < 0.01$.

Supplemental Figure 2



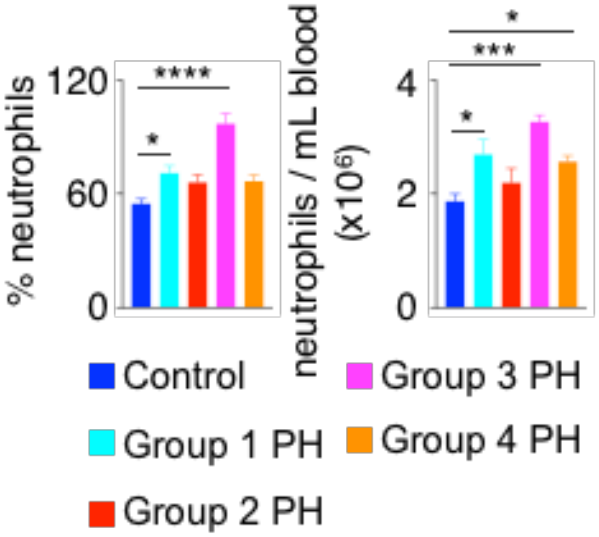
Supplemental Figure 2: Computational flow cytometry analyses show expression of cell surface markers in circulating leukocytes.
Multicolor flow cytometry was performed on blood samples obtained from patients with PAH. Computational flow cytometry analysis was performed to identify different circulating leukocyte populations based on the markers shown in this figure.

Supplemental Figure 3



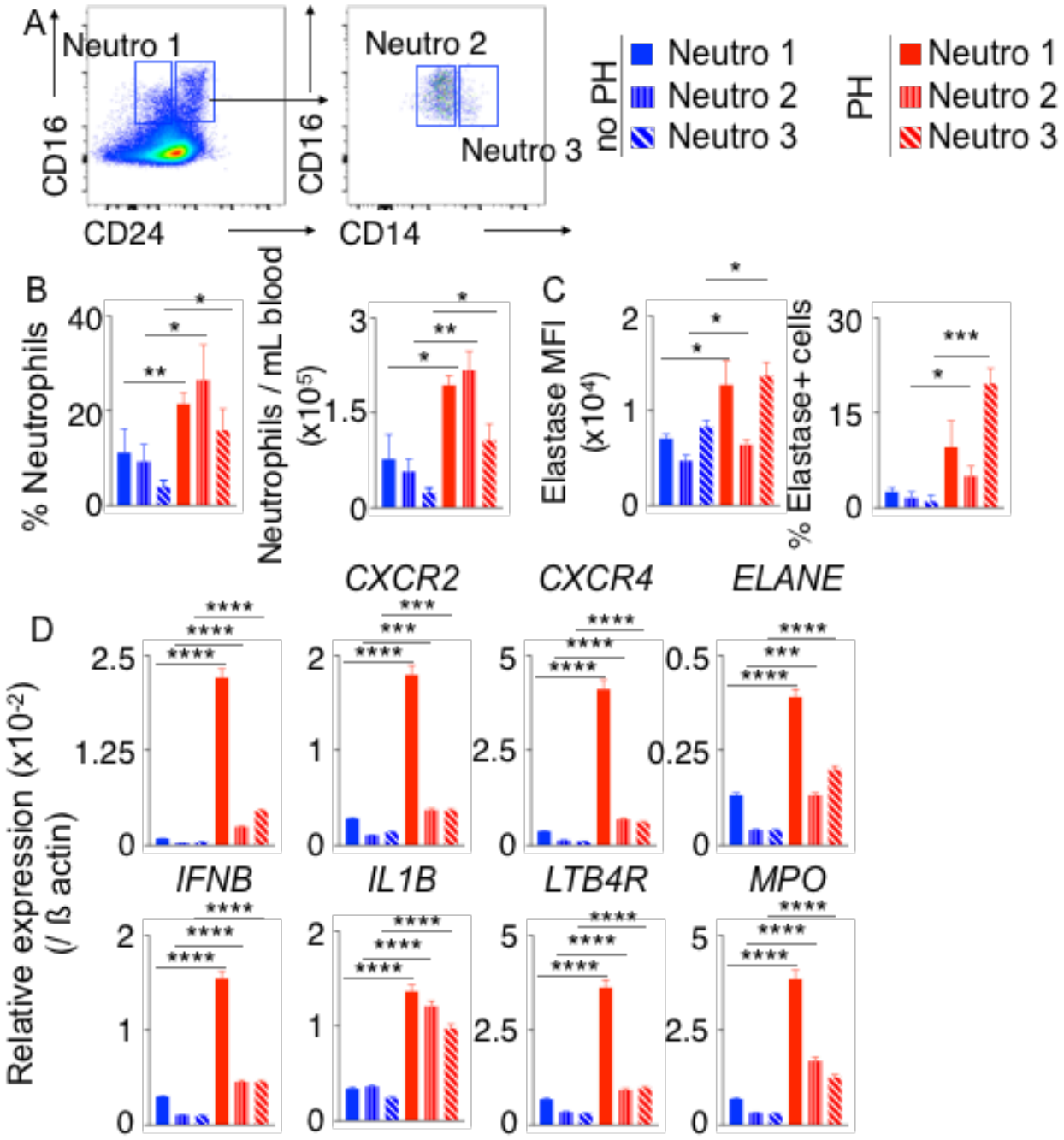
Supplemental Figure 3: Neutrophil population 1 expresses high levels of CD14, HLA-DR, CD56 and CD11c. A. The bh-SNE plot shows three distinct neutrophil populations. B. Computational flow cytometry analyses show expression of cell surface markers in neutrophil subpopulations.

Supplemental Figure 4



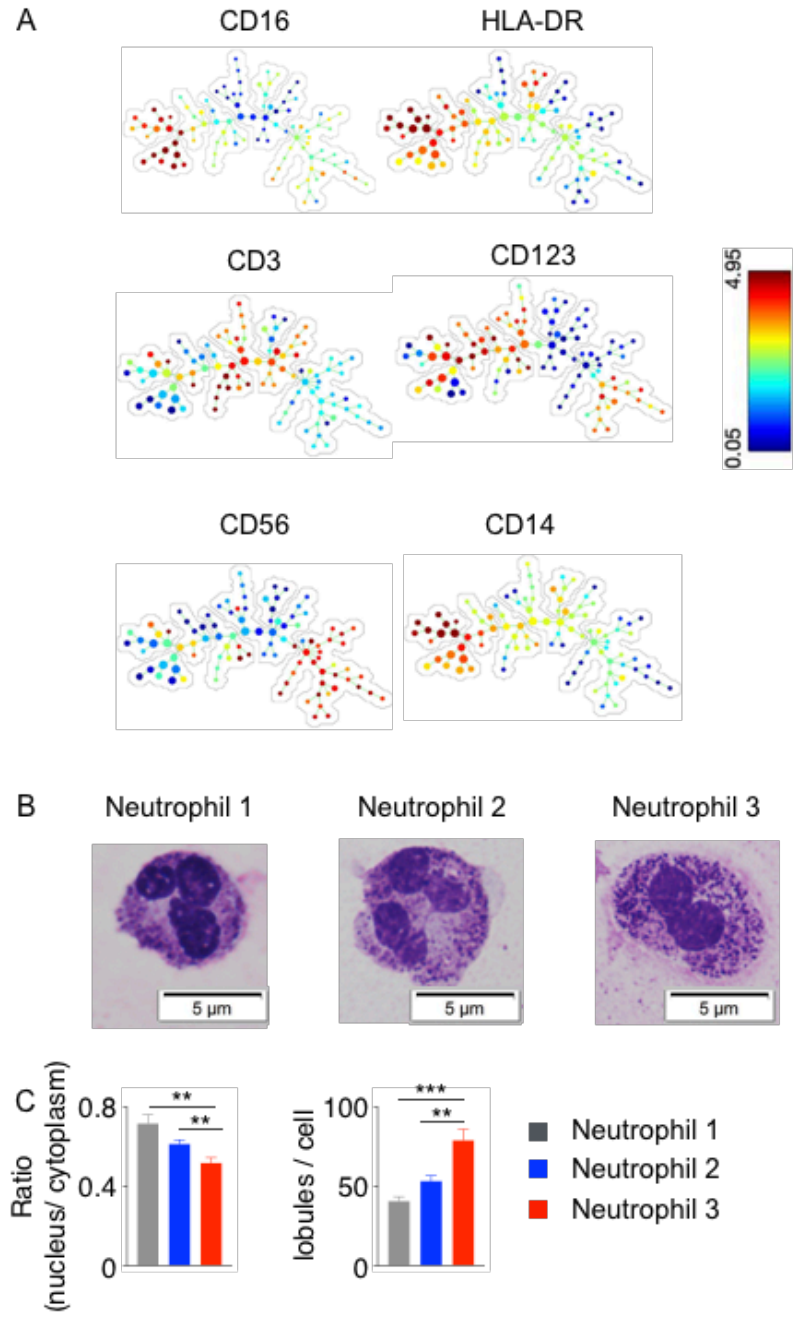
Supplemental Figure 4: Blood neutrophil levels in the different groups of PH. Blood neutrophils (percentage among myeloid cells and numbers) were enumerated by flow cytometry. n=5 samples per group. Mean ± s.e.m. * $P < 0.05$, *** $P < 0.05$, **** $P < 0.001$.

Supplemental Figure 5



Supplemental Figure 5: Neutrophil subpopulations in human PH lungs and blood have different pro-inflammatory properties. A. Flow cytometric dot plots showing the neutrophil subpopulations in the blood of PH patients. B. Enumeration (percentage among myeloid cells and numbers) of human circulating neutrophils by flow cytometry. C. Quantification of elastase and elastase^{+} neutrophils in the blood. D. Chemokine and pro-inflammatory cytokine mRNA expression was determined by RT-qPCR. n=5 samples per group. Mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$.

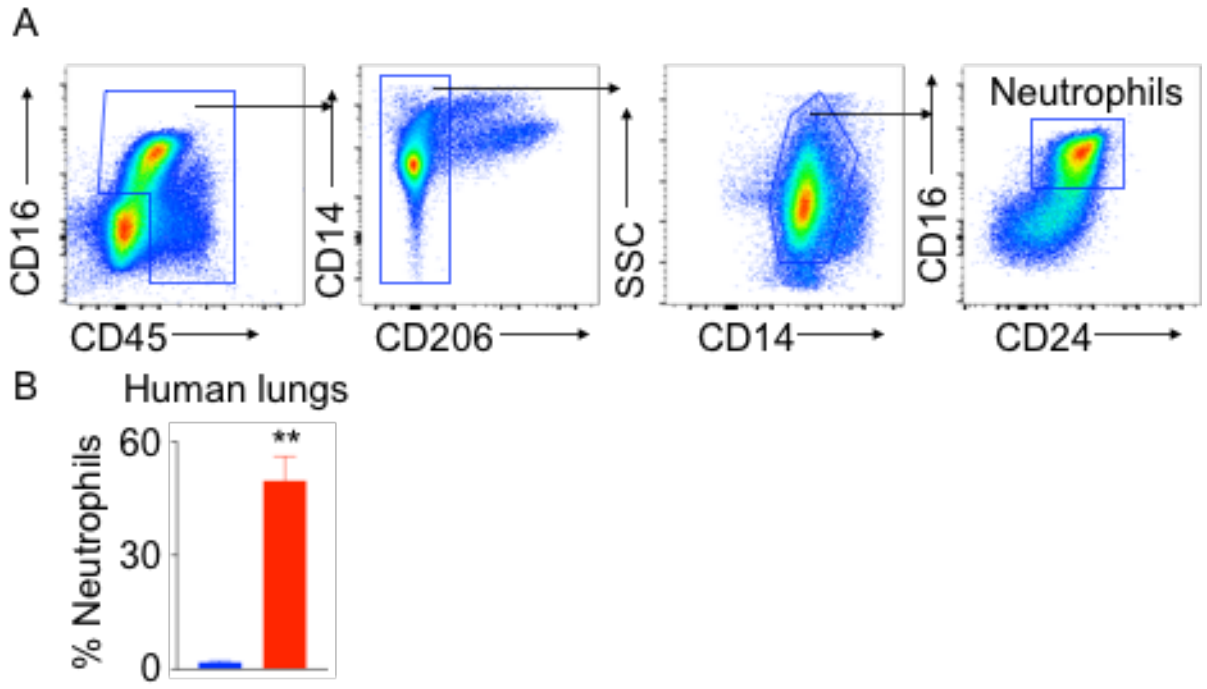
Supplemental Figure 6



Supplemental Figure 6: Computational flow cytometry and Giemsa staining analyses show phenotypical and morphological differences among the neutrophil subpopulations

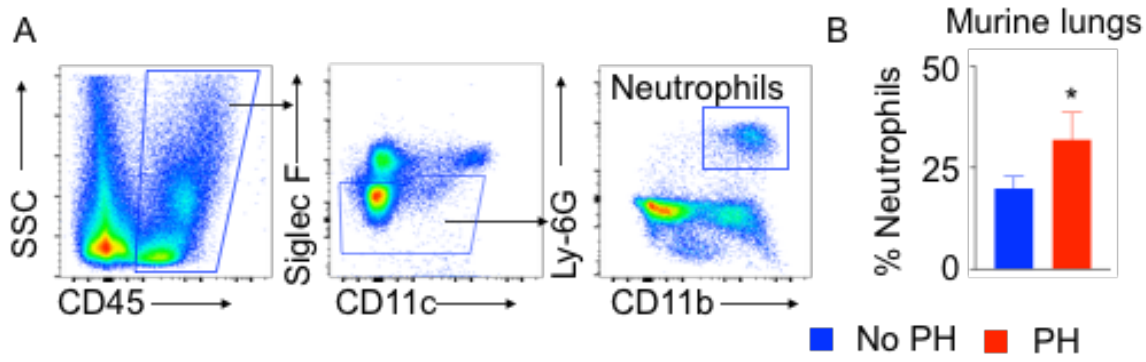
A. Spanning-tree Progression Analysis of Density-normalized Events (SPADE) was performed to determine the heterogeneity of circulating leukocytes in PAH patients. The expression of various cell surface markers is shown in leukocyte populations. B. Giemsa staining was performed on the three different subpopulations of human neutrophils. C. The ratio of nucleus to cytoplasmic volume and the number of lobules per cell has been determined. n=5 samples per group. Mean ± s.e.m. ** $P < 0.01$ and *** $P < 0.005$.

Supplemental Figure 7



Supplemental Figure 7: The frequency of neutrophils in the lungs of PH patients is increased. Lungs and blood of PAH patients and healthy controls were collected (n=7-8 for each group). A) Flow plots showing the gating strategy of human lung neutrophils. B) The percentage of human neutrophils among myeloid cells in the lungs was quantified. Mean ± s.e.m. ** $P < 0.01$.

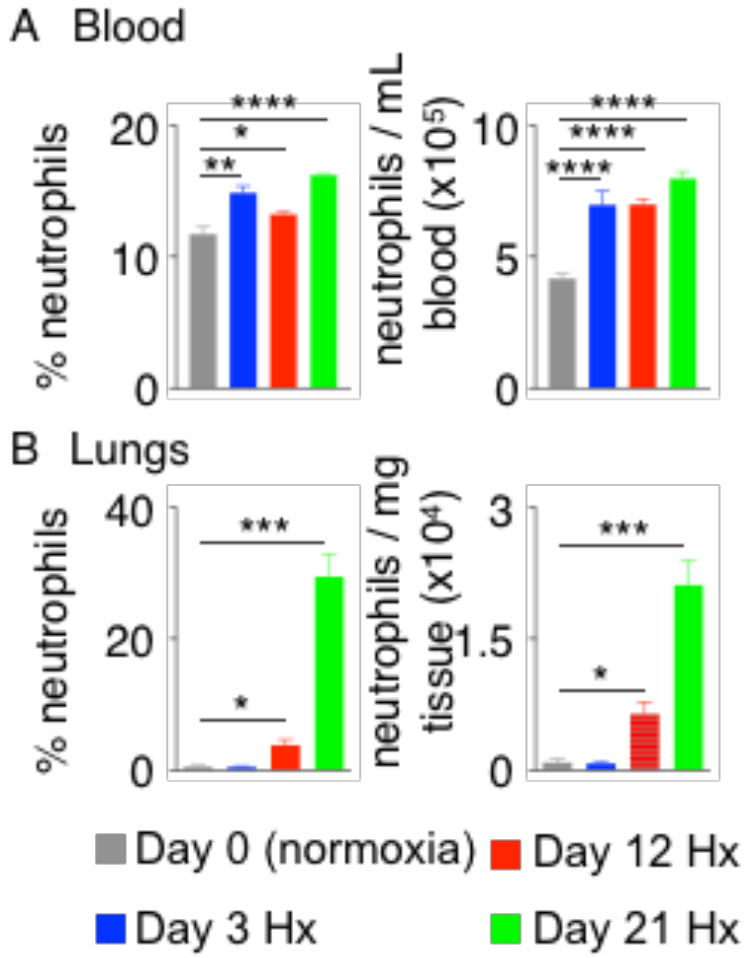
Supplemental Figure 8



Supplemental Figure 8: The frequency of neutrophils in the lungs of hypoxic mice is increased

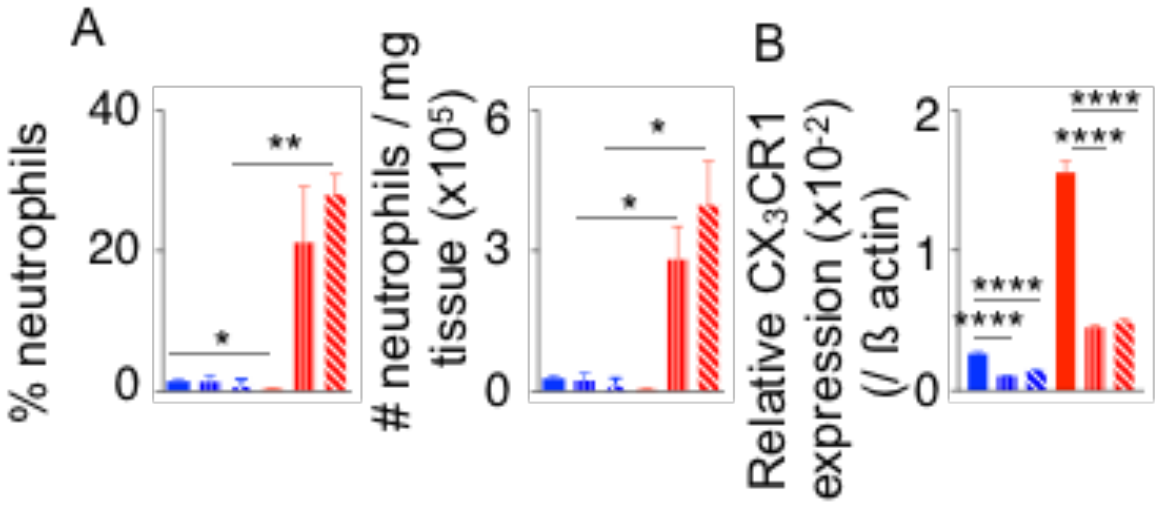
C57BL/6 mice were placed in a hypoxic chamber (10% O_2) (n=5 per group) for three weeks to induce PH. A) Flow plots showing the gating strategy of murine lung neutrophils. B) The frequency of murine lung neutrophils among myeloid cells was quantified. Mean ± s.e.m. * $P < 0.05$.

Supplemental Figure 9



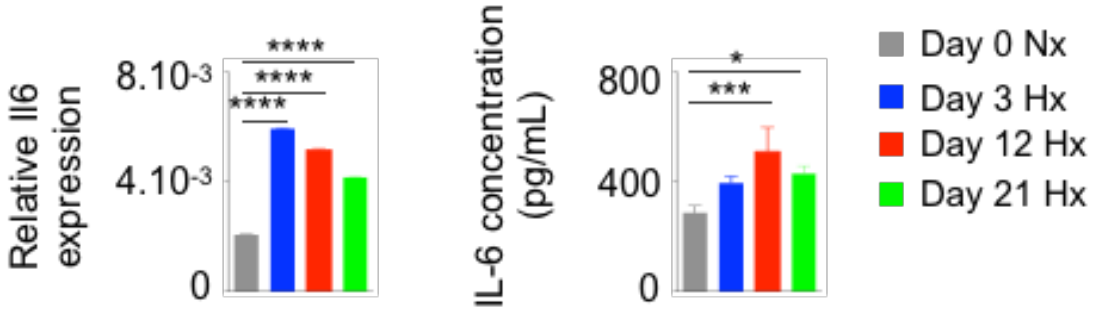
Supplemental Figure 9: Time course experiment of blood and lung neutrophils under hypoxia exposure. Blood (A.) and lung (B.) neutrophils (percentage among myeloid cells and numbers) were enumerated by flow cytometry 3, 12 and 21 days after hypoxia exposure. n=5 mice per group. Mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$.

Supplemental Figure 10



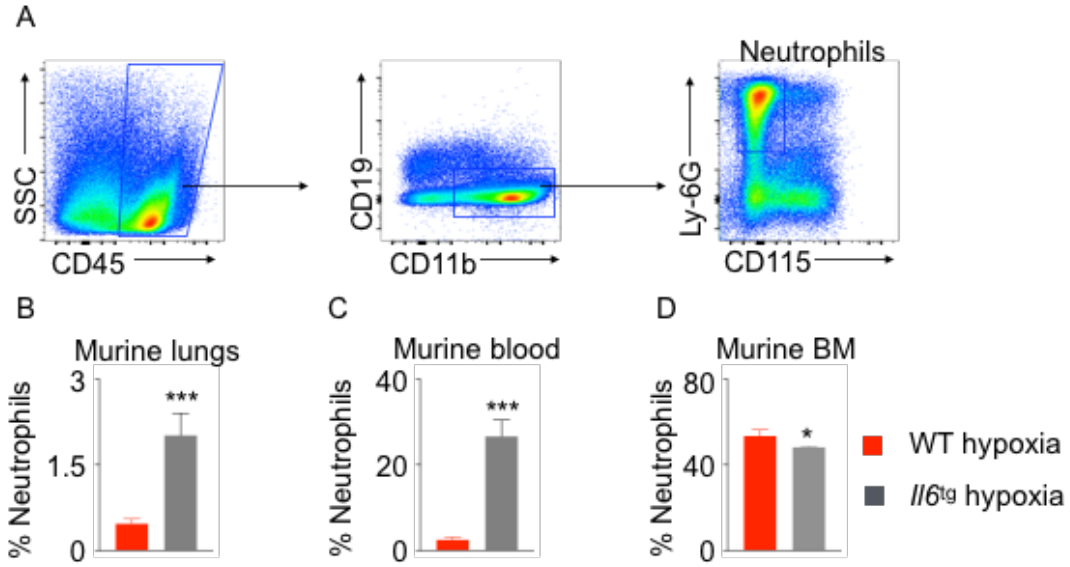
Supplemental Figure 10: Neutrophil subpopulations in human PH lungs express varying amounts of CX_3CR1. A. The frequency among myeloid cells and absolute numbers of the three neutrophil subpopulations were determined by flow cytometry in the lungs of PH patients. B. Relative *CX_3CR1* mRNA expression was determined by RT-qPCR in the three subpopulations of neutrophils in control and PH patients. n=5 samples per group. Mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.001$.

Supplemental Figure 11



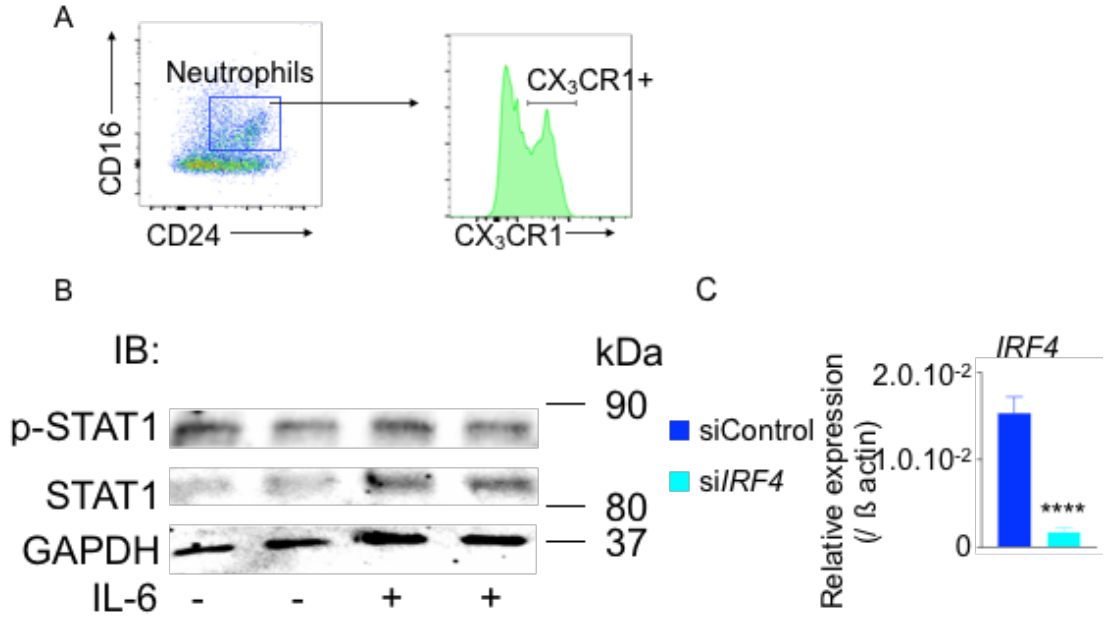
Supplemental Figure 11: IL-6 levels increase after hypoxia exposure.
C57BL/6 mice were placed in hypoxia for 3, 12 or 21 consecutive days. Day 0 represents the normoxic control mice**.** IL-6 levels were measured by RT-qPCR (left panel) and ELISA (right panel). n= 5 mice per condition. Mean ± s.e.m. * $P < 0.05$, *** $P < 0.005$, **** $P < 0.001$.

Supplemental Figure 12



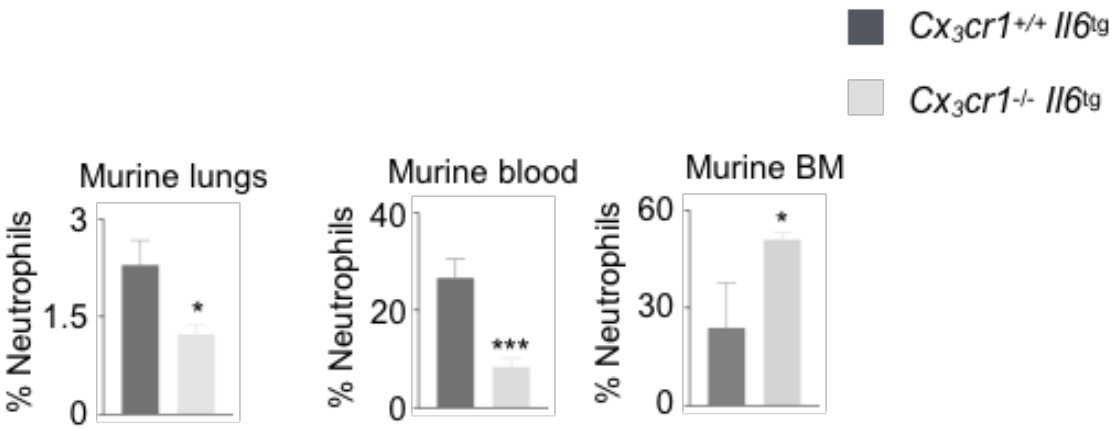
Supplemental Figure 12: IL6 over-expression in mice triggers neutrophilia in the lungs. *Il6*^{tg} and C57BL/6 mice were placed in a hypoxic chamber (10% O_2) for three weeks to induce PH. (n=5 per group). A) Flow plots showing the gating strategy of murine blood neutrophils. The frequency of neutrophils among myeloid cells was quantified in the lungs (B), blood (C) and BM (D) by flow cytometry. Mean ± s.e.m. * $P < 0.05$, *** $P < 0.005$.

Supplemental Figure 13



Supplemental Figure 13: pSTAT-1 levels do not change after IL-6 treatment.
A) Flow plots showing the gating strategy of differentiated HL-60 cells. B) Differentiated HL-60 cells were treated with 400 ng/mL of recombinant IL-6. p-STAT1 and STAT1 protein expression was determined by immuno blot. C) Differentiated HL-60 cells were treated with recombinant IL-6 (400 ng/mL) with control or *IRF4* siRNA. n=5 per group. *IRF4* expression was determined by qPCR. One of two independent experiment is shown. Mean ± s.e.m., **** P< 0.001.

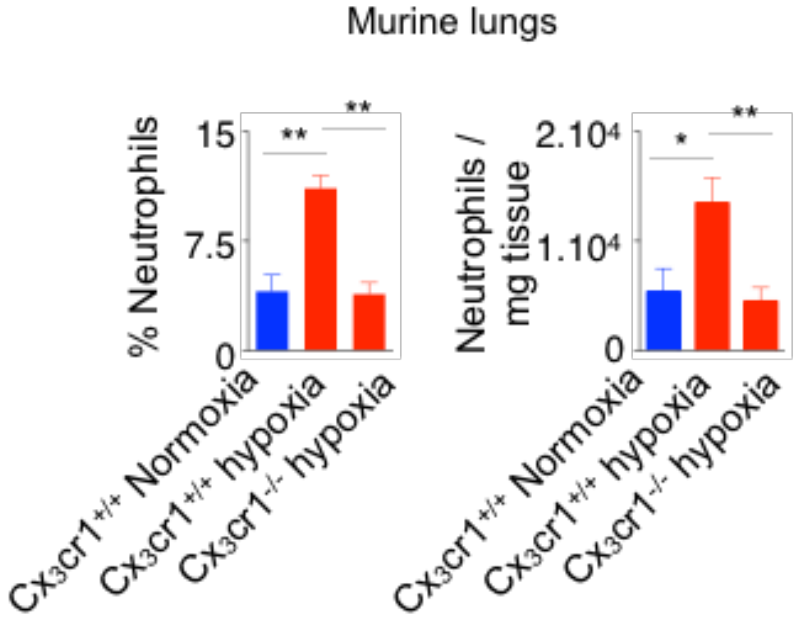
Supplemental Figure 14



Supplemental Figure 14: CX_3CR1 deficiency decreases the frequency of neutrophils in the lungs and increases their retention in the bone marrow of *Il6*^{tg} mice.

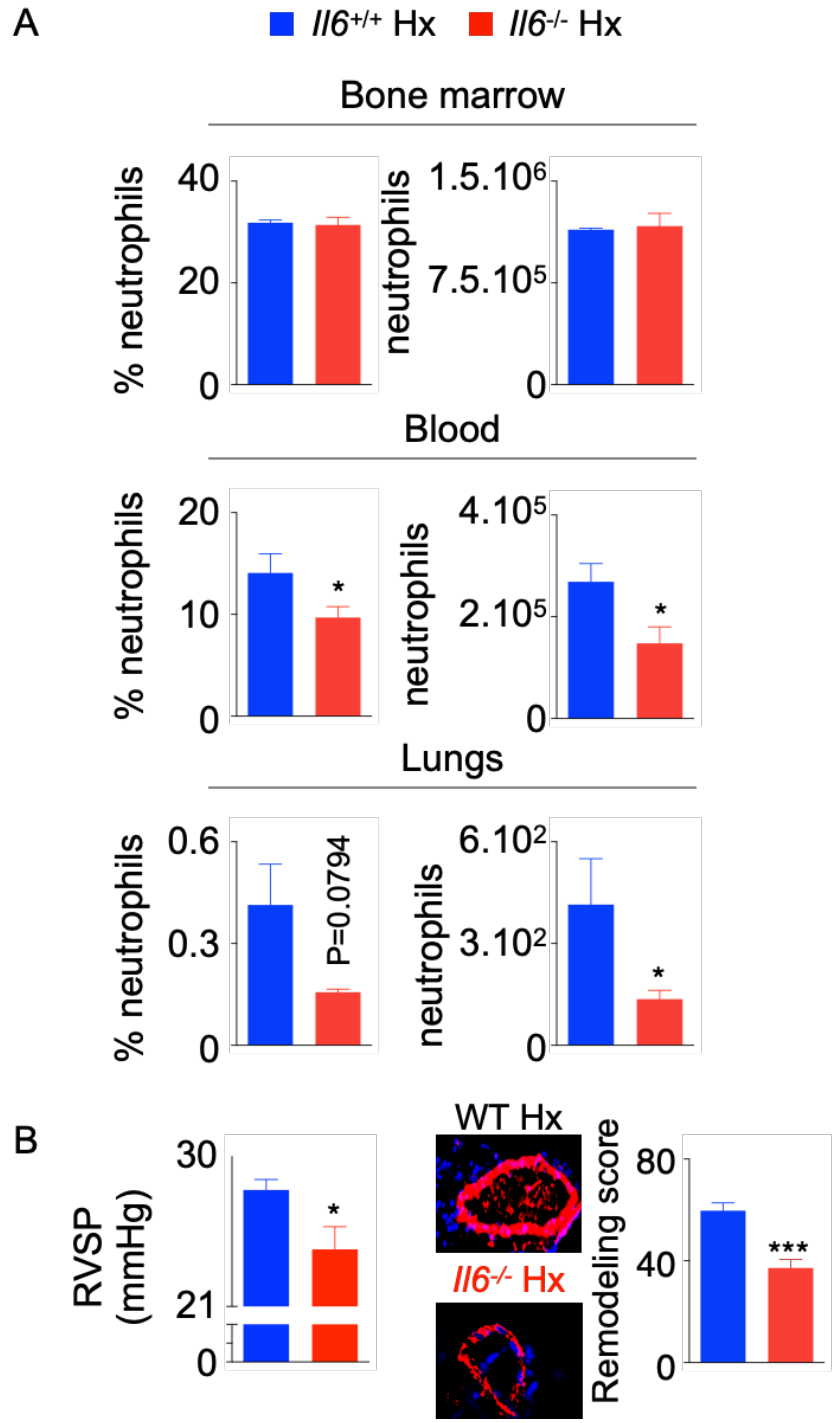
Cx₃cr1$^{+/+}$ *Il6*^{tg} and *Cx₃cr1*$^{-/-}$ *Il6*^{tg} mice were placed in hypoxic chambers (10% O_2) (n=5) for three weeks to induce PH. The frequency of lung, BM and blood neutrophils among myeloid cells was quantified by flow cytometry. Mean ± s.e.m. * $P< 0.05$.

Supplemental Figure 15



Supplemental Figure 15: *Cx₃cr1* deficiency decreases neutrophil rcruitment in the lungs. *Cx₃cr1*$^{+/+}$ and *Cx₃cr1*$^{-/-}$ mice were placed in hypoxic chambers (10% O_2) for three weeks to induce PH. The frequency and number of lung neutrophils among myeloid cells were quantified by flow cytometry. Mean ± s.e.m. n=5 per group, * P< 0.05, ** P<0.01.

Supplemental Figure 16



Supplemental Figure 16: *Il6* deficiency decreases neutrophil recruitment in the lungs and partially attenuates PH features.

Il6$^{+/+}$ and *Il6*$^{-/-}$ mice were placed in hypoxia for three weeks. A) The percentage and number of bone marrow, blood and lung neutrophils were assessed in these mice. B) Right ventricular systolic pressure and lung remodeling were assessed. n= 5 mice per condition. Mean ± s.e.m. * P< 0.05, ***P<0.001.

Supplemental Table 1

Patient (Lung)	Age (years)	Gender (M/F)	PH	mPAP (mm Hg)	PVR (WU)
PH1	21	M	Group 1 Idiopathic	69	19.52
PH2	56	F	Group 1 Scleroderma	65	7.44
PH3	53	F	Group 1 Scleroderma	53	8.79
PH4	42	M	Group 1 Scleroderma	30	0.9
PH5	50	F	Group 1 Idiopathic	59	8
PH6	53	F	Group 1 Scleroderma	44	5.14
PH7	60	F	Group 1 Scleroderma	45	5.5
PH8	44	F	Group 1 Scleroderma	27	3.1
PH9	72	F	Group 1 Idiopathic	42	12
PH10	23	F	Group 1 Idiopathic	44	8
PH11	74	F	Group 3	28	3.4
PH12	76	F	Group 3	55	7.26
PH13	73	F	Group 3	16	1.78
PH14	27	F	Group 3	45	5.91
PH15	85	M	Group 3	27	8
PH16	70	M	Group 3	47	7.54
CTL1	60	F			
CTL2	62	M			
CTL3	70	F			
CTL4	63	F			
CTL5	53	M			

Table S1. Demographics of PH patients used in this study. Lung tissue analysis: Five PAH patients (PH1-5) were prospectively enrolled at the time of death or transplant. Lungs tissues were processed and analyzed by flow cytometry. Paraffin-embedded lung tissues from those patients were further analyzed by *in situ* staining. **Blood analysis:** Peripheral venous blood from five additional PAH patients (PH6-10) and six WHO group 3 PH patients (PH11-16) prospectively enrolled in this study was used for comparison with peripheral venous blood from five healthy donors (CTL1-5) (Healthy donors: Mean age 61.6 $\pm$ 4.08 SEM, 60% female. PH patients: Mean age 54.9 $\pm$ 19.75 SEM, 75% female). Hemodynamic parameters are listed: Pulmonary capillary wedge pressure (PCW), mean pulmonary arterial pressure (mPAP), and pulmonary vascular resistance (PVR) are displayed.