Supporting material and methods

Human lungs and tissue preparation

Lung tissue was obtained over a three year period from 16 IPAH patients and 15 control lungs from the Division of Thoracic Surgery, Medical University of Vienna, Austria. Non-transplanted donor lungs that had been harvested for transplantation, but not implanted because of size-reduced lung transplantation, served as controls. All lungs were flushed via ante- and retrograde perfusion with Perfadex (XVIVO Perfusion, Göteborg, Sweden) to remove any residual blood. The protocol and tissue usage were approved by the institutional ethics committee (976/2010) and written patient consent was obtained before lung transplantation. Samples were coded for further analysis. Patient records including perioperative lab results as well as lung X-rays were checked carefully by two physicians to exclude the presence of any inflammatory conditions such as pneumonia, pulmonary tuberculosis or infections with pleural effusions.

A standardized protocol was established for the processing of tissue samples preceding the flow cytometric analysis to overcome any experimental bias. Two separate pieces of fresh lung (1-2cm^3) were taken from either peripheral or perihilar areas, weighed (generally 0.4-1g) and then digested at 37°C for 40 minutes with Collagenase type IV (200 ng/mL, Roche Applied science, Penzberg, Germany) and DNAse (200 ng/mL, Serva, Heidelberg, Germany) in RPMI media (Thermo Fisher Scientific, Vienna, Austria). Pulmonary arteries were identified in lung tissue by their anatomical position in relation to airways and carefully excised from the surrounding tissue with minimal handling (Fig S1). Isolated arteries were then weighed, digested and processed in the same fashion as lung tissue samples.

The cell-suspensions were filtered through a 100µm cell strainer and when necessary residual red blood cells were lysed with erythrolysis buffer (2.6 mM NH_4Cl, 0.09 M KCO_3, 0.6 M

EDTA). Cells were counted, stained with fixable viability stain (eBioscience, Vienna, Austria), washed and then fixed with 1% paraformaldehyde for 15 min on ice before being resuspended in MACS buffer (2 mM EDTA, 0.5% BSA).

Flow cytometry

To prevent nonspecific antibody binding the single-cell suspensions were first incubated with an Fc-receptor-binding antibody (eBioscience) for 5 min on ice. Cells were then stained using four different combinations of cell surface markers (Table S1) to identify each inflammatory cell population. Between 100,000 and 300,000 events were recorded on a LSRII flow cytometer (BD Biosciences) and analysed using either the FACSDiva (BD Biosciences, Vienna, Austria) or FlowJo (LLC, Ashland, Oregon) software by users blinded to disease condition. Cells were initially gated on FSC and SSC characteristics, duplexes were removed using FSC-A / FSC-H dot blot, dead cells were gated out using viability exclusion. Cells positive for the pan-leukocyte marker CD45 were taken for further analysis. The following antibody panels were used to detect cells of different lineages myeloid lineages (CD1a, CD11c, CD14, CD16, CD45, CD123, CD209 and HLA-DR; CD3, CD16, CD45, CD56, CD117, CD193 and CD203c), lymphocytes (CD3, CD4, CD8, CD19, CD45 and gdTCR). Full details are given in Tables S1 and S2. Data from individual lung tissue were averaged are presented as percentage of $CD45^+$ cells or as number of cells per mg of tissue as described in the text.

Blood sample preparation

A maximum of three mL whole blood was taken from an antecubital vein in EDTA-containing Vacutainer® tubes. Samples were processed within one hour. 300 µL of whole blood was transferred to FACS tube and 20 µL FC block added, 30 µL of pre-diluted antibody cocktail (FceRI, CD16, CD11c, CD123, CD303a, CD3, HLA-DR, CD304, CD14) was then

added and incubated for 20 min. Four mL of 1X FACS Lysing Solution (BD Bioscience) was then added followed by 100 µL 123count eBeads (eBioscience). Cells were centrifuged and washed once with MACS buffer before measurement on an LSRII Flow cytometer. Total number of cells per µL blood was calculated as follows: ((number cells*vol beads)/(number beads*vol blood))*conc beads per µL. The study was approved by the local ethics committee (23-408 ex 10/11). Written informed consent was obtained from all study participants. The preparation of blood, staining analysis was performed by investigators blinded to the patient´s clinical condition.

Biocomputational analysis

For bioinformatic analysis RStudio (Version 0.99.902) in combination with R (Version 3.3.1) was used. Results of different FACS panels were compared using the car package and plotted using the Scatterplot Matrix function; correlations were calculated using the Spearman's rank correlation. Percentage $CD45^+$ cells were converted to log odds ratios and the prcomp function was used to calculate the principal components. The first two principal components were plotted using the ggplot2 package. For heatmap representation, absolute cell counts per tissue weight were mapped to be between 0+d,1-d, where d=0.25*the smallest value in our dataset; the log Odds ratios were then plotted using the pheatmap package. Nonlinear-dimensionality reduction using t-distributed stochastic neighbour embedding (t-SNE) was performed in FlowJo. Briefly, the data from 4 IPAH and 4 donor lungs were cleaned up by exclusion of doublets, debris, and dead cells. Samples were individually down-sampled and $CD45^+$ events were concatenated and forwarded to t-SNE analysis.

Immunofluorescence staining and quantification

Immunofluorescent staining was performed on 3 µm thick paraffin-embedded lung sections. Sections were deparaffinised and antigen retrieval was conducted with DAKO solution (pH9,

0.05% Tween) for 20 min at 95°C in water bath. Tissue was blocked for 1 h with 3% BSA before incubation with primary antibodies at 4°C overnight. The following antibody dilutions were applied CD123 (IL3Rα), 1:200 (V-18, Santa Cruz Biotechnology, Santa Cruz, USA); CD304, 1:100 (A-12, Santa Cruz Biotechnology)), TCRγ, 1:50, (biorbyt, Cambridge, UK), Smooth muscle actin (SMA), 1:100 (Everest Biotech, Bicester, UK). Negative controls were performed omitting the primary antibody or isotypes. After incubation with primary antibodies, the tissue was washed with 0.1 % BSA in PBS and incubated with the following secondary antibodies; goat-anti-Rabbit Alexa Fluor® 555 (CD123), donkey anti-Goat Alexa Fluor® 647 (SMA) or ImmPRESS reagent peroxidase (CD304/TCRγ; Vector Laboratories, Peterborough, UK) in combination with the Tyramide Alexa Fluor® 488 Signal Amplification Kit (Thermo Fischer Scientific) for 1 h. Tissue was then mounted with fluorescence Vectashield mounting medium (Vector Laboratories) including DAPI. Confocal images were made with a laser-scanning confocal microscope, LSM 510 Meta (Zeiss, Gottingen, Germany), with the following Ex/Em settings: 405/BP420–480 (DAPI); 488/BP420-480 (Alexa Fluor 488); 543/BP530-600 (Alexa Fluor 555) and 633/LP650 (Alexa Fluor 647). The images with 2048 × 2048 pixels in size were taken with a Zeiss 40 × oil immersion objective with 1.4 NA. To quantify pDC and gdT cells, multiple 10x images (8-11 per lung) containing a pulmonary artery where taken and the number of positive cells enumerated.

Fig. S1: *Pulmonary arterial isolation procedure*

Pulmonary arteries(*) were identified under a stereomicroscope due to their close vicinity to airways(§). Arteries were exposed by gradual removal of lung tissue until ~1-2cm of artery was visible. The artery was then removed en-bloc including side branches and forwarded to cell isolation. Scale bar represents 1cm.

Fig. S2: *Lung morphology in donor and IPAH samples*

Representative images of sections derived from explanted lung samples (n=4). Magnified areas containing pulmonary vessels are also shown.

Fig. S3: *Correlation plots of cell populations measured in different flow cytometry panels*

A) Total $CD45^+$ cells, B) PMN: polymorphonuclear neutrophils, C) CD3+ T cells, D) Mac: macrophages, and E) Mast cells. P values and rho (r) estimates derived from Spearman's rank correlation are shown for each correlation.

Fig. S4: *Correlation analysis of individual cell populations in matched lung samples*

Analysed cell populations in the two separate pieces of lung were compared by Spearman's rank correlation. P values and rho (r) estimates derived from Spearman's rank correlation are shown.

Fig. S5: *Abundance of cell populations excluded from global principal component analysis*

Scatterplot and boxplot overlays of cell populations in IPAH and donor lungs as analysed by flow cytometry. NK, natural killer cells; NKT, natural killer T cells. Boxplots show median and interquartile range.

Fig. S6: *Patient-to-patient variability in $CD45^+$ cell populations*

Heatmap representation presenting the patient-to-patient variability of the log odds ratio derived from the percentage $CD45^+$ data for 18 individual cell populations. Blue colours indicate highest abundance, red lowest for a single cell type. PMN, polymorphonuclear

neutrophils; Macs, Macrophages; Monos, monocytes; DC, dendritic cells; pDC, plasmacytoid DC; gd gamma delta.

Fig. S7: *Euclidean clustering of individual IPAH and Donor samples*

Heatmap representation using Euclidean clustering of the log odds ratio derived from number of cells per mg tissue data for the 21 individual cell populations. Blue colours indicate highest abundance, red lowest for a single cell type. Monos, monocytes; DC dendritic cells, Macs, Macrophages; CTL, cytotoxic T cell; gd gamma delta; PMN, polymorphonuclear neutrophils.

Fig. S8: *Abundance of remaining cell population in lung samples*

Abundance of cell populations not included in Fig. 4 as determine by flow cytometric analysis of IPAH and donor lungs. DC, dendritic cells; PMN, polymorphonuclear neutrophils. Boxplots show median and interquartile range, statistical significance between groups was determined by the Wilcoxon rank sum test.

Fig. S9: *Relative proportions of $CD45^+$ cells in lung samples*

The relative proportions of multiple cell populations in IPAH and donor lungs. DC, dendritic cells; pDC; plasmacytoid dendritic cells; PMN, polymorphonuclear neutrophils. Boxplots show median and interquartile range, statistical significance between groups was determined by the Wilcoxon rank sum test.

Fig. S10: *Abundance of cell population in isolated pulmonary arteries*

Remaining cell populations not included in Fig. 5 as measured by flow cytometric analysis in IPAH and donor samples. DC, dendritic cells; PMN, polymorphonuclear neutrophils. Boxplots show median and interquartile range, , statistical significance between groups was determined by the Wilcoxon rank sum test.

Table S1: *Antibodies used in flow cytometric analysis*

Antigen	Label	Company	Clone	Isotype	Dilution	FACS Panel
CD1a	AF700	Biolegend	HI149	Ms IgG1κ	10	2
CD203c	PerCp-Cy5.5	Biolegend	HLDA8	Ms IgG1κ	20	3
CD3	FITC	eBioscience	UCHT1	Ms IgG1κ	20	1
CD3	ef450	eBioscience	UCHT1	Ms IgG1κ	20	3
CD4	PE-Cy7	eBioscience	SK3	Ms IgG1κ	20	1
CD8	APC-Cy7	BD Bioscience	SK1	Ms IgG1κ	20	1
CD11c	PerCP-Cy5.5	eBioscience	3.9	Ms IgG1κ	10	2
CD14	ef450	eBioscience	61D3	Ms IgG1κ	20	2
CD14	APC-AF780	eBioscience	61D3	Ms IgG1κ	20	4
CD16	PE-Cy7	eBioscience	eBioCB16	Ms IgG1κ	20	3
CD16	Ef450	eBioscience	eBioCB16	Ms IgG1κ	20	4
CD19	APC	eBioscience	HIB19	Ms IgG1κ	20	1
CD25	ef450	eBioscience	BC96	Ms IgG1κ	20	1
CD45	FITC	eBioscience	HI30	Ms IgG1κ	100	2 / 3
CD45	PerCP-Cy5.5	eBioscience	HI30	Ms IgG1κ	100	1 / 4
CD56	APC-ef780	eBioscience	CMSSB	Ms IgG1κ	20	3
CD117	APC	eBioscience	YB5.B8	Ms IgG1κ	10	3
CD117	PE-Cy7	eBioscience	YB5.B8	Ms IgG1κ	10	4
CD123	PE-Cy7	ebioscience	6H6	Ms IgG1κ	20	2
CD193	PE	eBioscience	5E8-G9-B4	Ms IgG2bκ	20	3
CD209	AF647	Biolegend	9E9A8	Ms IgG1κ	20	2
HLA-DR	APC-ef780	eBioscience	LN3	Ms IgG1κ	200	2
γδTCR	PE	eBioscience	B1.1	Ms IgG1κ	20	1

Table S2: *Cell population markers used in flow cytometric analysis*

Lineage		Cell type	FSC	SSC	Surface Markers						
Lymphoid		T cells	Low	Low	$CD45^+$	$CD3^+$			$CD19^-$		
		$CD4^+$ T cells	Low	Low	$CD45^+$	$CD3^+$	$CD4^+$	$CD8^-$			
		$CD8^+$ T cells	Low	Low	$CD45^+$	$CD3^+$	$CD4^-$	$CD8^+$			
		γδ T cells	Low	Low	$CD45^+$	$CD3^+$	$CD4^-$	$CD8^-$	$gdTCR^+$		
		NKT cells	Low	Low	$CD45^+$	$CD3^+$				$CD56^+$	
		NK cells	Low	Low	$CD45^+$	$CD3^-$				$CD56^+$	
		B cells	Low	Low	$CD45^+$	$CD3^-$			$CD19^+$		
Myeloid	Granulocytes	Basophils (Baso)	Low	Low	$CD45^+$	$HLA\text{-}DR^-$		$CD11c^-$	$CD123^+$		
		Eosinophils (EOS)	Med	High	$CD45^+$	$HLA\text{-}DR^-$	$CD14^-$	$CD16^-$	$CD193^+$		
		Neutrophils (PMN)	Med	Med	$CD45^+$	$HLA\text{-}DR^-$	$CD14^-$	$CD16^+$	$CD193^-$		
		Mast cells	Med	Med	$CD45^+$			$CD16^-$	$CD193^-$	$CD117^+$	$CD203c^+$
	Macrophages (Macs)	Total Macs	High	High	$CD45^{high}$	$HLA\text{-}DR^{+/-}$				$CD1a^{+/-}$	
		Activated $HLA\text{-}DR^+$ Macs	High	High	$CD45^{high}$	$HLA\text{-}DR^+$	$CD14^+$	$CD16^+$		$CD1a^+$	
		Non-activated $HLA\text{-}DR^+$ Macs	High	High	$CD45^{high}$	$HLA\text{-}DR^+$	$CD14^+$	$CD16^+$		$CD1a^-$	
		Activated $HLA\text{-}DR^-$ Macs	High	High	$CD45^{high}$	$HLA\text{-}DR^-$	$CD14^+$	$CD16^+$		$CD1a^+$	
	Monocytes (Monos)	Classical Monos (Classic)	Med	Med	$CD45^{high}$		$CD14^{++}$	CD16-			
		Intermediate Monos (Int.)	Med	Med	$CD45^{high}$		$CD14^{++}$	$CD16^+$			
		Non-classical Monos (Non class.)	Med	Med	$CD45^{high}$		$CD14^+$	$CD16^{++}$			
	Dendritic cells (DC)	Plasmacytoid dendritic cells (pDC)	Low	Low	$CD45^{high}$	$HLA\text{-}DR^+$	$CD14^-$	$CD11c^-$	$CD123^+$		
		$CD11c^+$ $CD209^+$ DC	Low/Med	Low/Med	$CD45^{high}$	$HLA\text{-}DR^+$	$CD14^-$	$CD11c^+$	$CD209^+$		
		Activated $CD11c^+$ $CD209^+$ DC	Low/Med	Low/Med	$CD45^{high}$	$HLA\text{-}DR^+$	$CD14^-$	$CD11c^+$	$CD209^+$	$CD1a^+$	
		$CD11c^-$ $CD209^+$ DC	Low/Med	Low/Med	$CD45^{high}$	$HLA\text{-}DR^+$	$CD14^-$	$CD11c^-$	$CD209^+$		
		$CD11c^+$ $CD209^-$ DC	Low/Med	Low/Med	$CD45^{high}$	$HLA\text{-}DR^+$	$CD14^-$	$CD11c^+$	$CD209^-$		
		Activated $CD11c^+$ $CD209^-$ DC	Low/Med	Low/Med	$CD45^{high}$	$HLA\text{-}DR^+$	$CD14^-$	$CD11c^+$	$CD209^-$	$CD1a^+$	

Table S3: *Patients characteristics of samples used in analysis of peripheral blood*

Characteristics	Donor	IPAH
Total samples	18	12
Age (y)	56.6±16.3	68.7±9.7*
Gender(m/f)	6/12	3/9
Height (cm)	167±8	165±8
Weight (kg)	66.7±9.4	79.2±15.5*
mPAP (mmHg)	-	45.2±13.9
PVR (dyn)	-	785±488
6MWT (m)	-	318±128
NYHA class	-	II-III
pro BNP [pg/ml]	94.1±129	1522±1347
CRP [mg/dl]	1.8±1.1	5.3±5.1*

*p≤0.05; years (y), male (m), female (f) mPAP: mean Pulmonary arterial pressure; PVR: pulmonary vascular resistance; 6MWD: 6 min walking distance; NYHA: New York Heart Association; BNP: b-type natriuretic peptide; CRP: C- reactive protein. Mean±SD