

1 **Online Supplementary data to:**

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3 **Loss of Immune Homeostasis in Patients with Idiopathic Pulmonary Arterial**
4 **Hypertension**

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6 Peter Heukels et al

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32 **online supplementary methods**

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34 **Human flow cytometry procedures**

35 Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes (BD Vacutainer K2E).
36 Peripheral blood mononuclear cells (PBMCs) and plasma were obtained, processed and stored
37 according to standard protocols.¹

38 PBMCs were stained for extra- and intracellular markers (**online supplementary table 1**). To
39 prevent non-specific labeling Fc-block (Anti-Mouse CD16/CD32 Fc-Block) was used. Fixable Viability
40 Dye eFluor 506 (eBiosciences) was applied as a live-dead marker. Flow cytometry procedures for B-cell
41 staining have been described previously². Cells for the T-cell staining were first incubated in MACS
42 buffer (0.5% BSA + 2mM EDTA in PBS) with fluorescent antibodies against chemokine receptors for 60
43 minutes at 4°C. A second extracellular incubation step was performed for antibodies with Brilliant
44 Violet (BV) conjugates in Brilliant Stain-buffer (BD Biosciences, cat#563794). After fixation and
45 permeabilization, cells were incubated with a forkhead box P3 (FOXP3)-specific antibody in
46 permeabilization buffer for 60 minutes at 4°C. Live cells (>200,000) were acquired and data were
47 analyzed by FACS Flow-Jo software.

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49 **mouse experiments and procedures**

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51 **Genotyping and inducing pulmonary injury**

52 CD19-hBtk³ on a mixed background (Fvb × 129/Sv × C57BL/6J) were backcrossed on C57BL/6J for > 10
53 generations. Genotyping was performed by polymerase chain reaction (PCR), as previously described.⁴
54 Wild-type mice used for the experiments are non-transgenic littermates. Mice were bred and kept at
55 specified pathogen-free conditions in the Erasmus MC experimental animal facility. All experimental
56 protocols have been reviewed and approved by the Erasmus MC Committee of animal experiments.
57 To induce pulmonary injury, bleomycin-hydrochloride was administered intra tracheally in 8-10 week
58 old mice (0,04U/80 µl saline) or saline as a control as previously described.⁵ Mice were sacrificed 21
59 days and 70 days after bleomycin exposure.

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61 **Right ventricular systolic pressure (RVSP) and lung tissue elastance**

62 Mice were anaesthetized with isoflurane and right ventricular pressures were recorded using right
63 heart catheterizations (mikro-tip catheter 1,4F, Millar instruments, model SPR-671) and analyzed with
64 WinDaq Data acquisition software. Lung tissue elastance was measured with a flexiVent FX system as
65 previously described.⁶ Data was analyzed with flexiWare 7 software.

66

67 Flow cytometric procedures

68 Preparations of single-cell suspensions of MLN using standard procedures. Monoclonal antibodies are
69 listed in **online supplementary table 1**. For intracellular staining, cells were fixed in Cytofix/Cytoperm
70 and permeabilized, and then stained in Perm/Wash buffer (BD Bioscience). All measurements were
71 performed on a LSRII flow cytometer (BD Bioscience), and results were analyzed using FlowJo software.
72

73 Immunohistochemistry

74 Immunohistochemical analyses and staining were performed according to standard procedures.⁷ Used
75 antibodies are listed in **online supplementary table 1**. After staining, tissue sections were embedded
76 in Kaiser glycerol gelatin (Merck). Pulmonary vascular remodeling was studied by quantification of
77 intraacinar pulmonary vessels containing α -smooth muscle actin (α -SMA)-positive cells in their walls.
78 Vessels between and 15 and 50 μ m external diameter and located in normal lung tissue were assessed.
79 To assess the presence of self-reactive antigens in serum of CD19-hBTK and control mice, serum was
80 stored and frozen until further use. Serum was subjected to cryo-sections of lungs of RAG^{-/-} mice
81 (lacking mature B and T lymphocytes). Detection Antibodies against IgG and IgM are listed in **online**
82 **supplementary table 1**. Micrographs were made using a DM LB light microscope (Leica), a DFC500
83 camera (Leica), and Imaging for Windows Version 1.0 software (Kodak).

84

85 The Fulton index

86 Hearts were excised and dissected to determine the ratio of right ventricular to left ventricular and
87 septal weight [RV/(LV + S)].

88

89 Hydroxy proline assay

90 Whole left lung homogenates were analyzed by quantitative hydroxyproline assay. The left lung
91 homogenate was hydrolyzed in 6M HCl at 95°C for 20 hours. Hydroxyproline was oxidized with
92 chloramine t, and visualized with Erlich's reagent (4-DMAB, isopropanol and perchloric acid)
93 measured at a microplate reader at 560nm.

94

95 HEp-2

96 To assess the presence of self-reactive antigens in serum of CD19-hBTK and control mice, serum was
97 stored and frozen until further use. Serum samples (1/50 diluted) were incubated for 1 hour on
98 Kallestad human epithelial cell (HEp-2) slides (Bio-Rad Laboratories). As detection antibodies Ig
99 F(ab')₂ fragments were applied to the HEp2 slides (**online supplementary table 1**). The fluorescence

100 intensity of HEp2 slides was evaluated using a LSM 311 META confocal fluorescence microscope
101 (Zeiss) and LSM Image Browser Version 4.2.0.12 software (Zeiss)

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103 **α -SMA area to total artery area**

104 Percentage α -SMA area to total artery area was evaluated using NanoZoomer 2.0-HT slide scanner
105 (Hamamatsu) and NDP.view 2.7.25 (Hamamatsu). Photos were analysed using Adobe Photoshop
106 2021 in an automated and thus independent manner.

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108 **Total fibrosis score**

109 A pathologist (blinded for treatment) scored the Ashcroft scale (grade 1-8)⁸ and the percentage of
110 lung involvement (grade 1-5; 1 =0-10% to 6 = 75-100% of total lung involvement). The Total Fibrosis
111 score (TFS) is the product of Ashcroft scale and lung involvement and was previously described.⁹

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113 1. Heukels P, van Hulst JAC, van Nimwegen M, Boorsma CE, Melgert BN, van den Toorn LM, *et al.* Fibrocytes are increased in lung and peripheral blood of patients with idiopathic
114 pulmonary fibrosis. *Respir Res* 2018, **19**(1): 90.

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117 2. Corneth OBJ, Verstappen GMP, Paulissen SMJ, de Bruijn MJW, Rip J, Lukkes M, *et al.*
118 Enhanced Bruton's Tyrosine Kinase Activity in Peripheral Blood B Lymphocytes From Patients
119 With Autoimmune Disease. *Arthritis Rheumatol* 2017, **69**(6): 1313-1324.

120

121 3. Maas A, Dingjan GM, Grosveld F, Hendriks RW. Early arrest in B cell development in
122 transgenic mice that express the E41K Bruton's tyrosine kinase mutant under the control of
123 the CD19 promoter region. *J Immunol* 1999, **162**(11): 6526-6533.

124

125 4. Middendorp S, Dingjan GM, Maas A, Dahlenborg K, Hendriks RW. Function of Bruton's
126 tyrosine kinase during B cell development is partially independent of its catalytic activity. *J*
127 *Immunol* 2003, **171**(11): 5988-5996.

128

129 5. Kim SN, Lee J, Yang HS, Cho JW, Kwon S, Kim YB, *et al.* Dose-response Effects of Bleomycin on
130 Inflammation and Pulmonary Fibrosis in Mice. *Toxicol Res* 2010, **26**(3): 217-222.

131

132 6. McGovern TK, Robichaud A, Fereydoonzad L, Schuessler TF, Martin JG. Evaluation of
133 respiratory system mechanics in mice using the forced oscillation technique. *J Vis Exp*
134 2013(75): e50172.

135

- 136 7. GeurtsvanKessel CH, Willart MA, Bergen IM, van Rijt LS, Muskens F, Elewaut D, *et al.*
137 Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of
138 influenza virus-infected mice. *J Exp Med* 2009, **206**(11): 2339-2349.
- 139
140 8. Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary
141 fibrosis on a numerical scale. *J Clin Pathol* 1988, **41**(4): 467-470.
- 142
143 9. Borensztajn K, Bresser P, van der Loos C, Bot I, van den Blink B, den Bakker MA, *et al.*
144 Protease-activated receptor-2 induces myofibroblast differentiation and tissue factor up-
145 regulation during bleomycin-induced lung injury: potential role in pulmonary fibrosis. *Am J*
146 *Pathol* 2010, **177**(6): 2753-2764.
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165 **online Supplementary Table 1** Overview of extra- and intracellular antibodies use for human and
 166 mouse experiments.

marker	conjugate	company	Cat#	intra/extracellular	dilution
HUMAN					
antibodies used for flow cytometry					
IgG	FITC	BD	555786	extracellular	1:20
Btk	PE	BD	611117	intracellular	1:5
IgM	Bio	BD	555781	extracellular	1:20
CD19	PerCP-Cy5.5	BD	332780	extracellular	1:400
CD38	APC	BD	560980	extracellular	1:10
IgD	APC-H7	BD	561305	extracellular	1:10
CD27	BV421	BD	562513	extracellular	1:80
CD24	BV711	BD	563401	extracellular	1:40
CD3	AF700	eBioscience	56-0038-42	extracellular	1:40
CXCR5	PerCP5.5	BD	562781	extracellular	1:20
CD3	APC ef780	eBioscience	47-0038-42	extracellular	1:100
CD4	AF700	eBioscience	E08948-1631	extracellular	1:100
CD45RA	BV650	BD	563963	extracellular	1:40
PD1	BV786	BD	563789	extracellular	1:20
FoxP3	PE	eBioscience	12-4777-42	intracellular	1:20
CXCR3	BV711	BD	563156	extracellular	1:20
CCR4	FITC	BD	FAB1567F	extracellular	1:20
CCR6	APC	BD	560619	extracellular	1:5
CXCR5	PerCP5.5	BD	562781	extracellular	1:20
HEp-2 antibodies					
Anti-Mouse F(ab') IgG	Cy3	Jackson IR	115-166-003		
MOUSE					
antibodies used for flow cytometry					
GL7	FITC	BD	553666	extracellular	1:2000
CD95	PE-TxR	BD	562499	extracellular	1:400
IgM	Pe-Cy7	eBioscience	25-5790-82	extracellular/ intracellular	1:500
IgD	APC	eBioscience	17-5993-82	extracellular/ intracellular	1:1280
CD19	Af700	eBioscience	56-0193-82	extracellular	1:50
CD138	BV605	BD	563147	extracellular	1:400
CD3	PE-CF594	BD	562286	extracellular	1:100
CD4	Af700	eBioscience	56-0041-82	extracellular	1:200
MHC II	FITC	BD	553565	extracellular	1:200
PD-1	PE	BD	551892	extracellular	1:100
CD3	PE-CF594	BD	562286	extracellular	1:100
CD40L (CD154)	PerCP-eFl710	eBioscience	46-1541-82	extracellular	1:100
CXCR5	biotin	BD	551960	extracellular	1:50
ICOS	APC	eBioscience	17-9949-82	extracellular	1:1600

CD4	AF700	eBioscience	56-0041-82	extracellular	1:400
CD11c	APC-eFl750	eBioscience	47-0114-82	extracellular	1:200
CD11b	eFl450	eBioscience	48-0112-82	extracellular	1:200
PD-L1	BV711	BD	563369	extracellular	1:100
HEp-2 antibodies					
Anti-Human F(ab') IgG	Cy3	Jackson IR	109-166-003		
Immunohisto- chemistry Lung					
Anti- α SMA	PE	R&D	IC1420P		
Anti-PE	AP	Rockland	600-105-387		
Anti-B220	Unlabeled	Bioceros			
Anti-Rat	AP	Sigma	A8438 – 1ML		
Anti-hCD3	Unlabeled	DAKO	A0452		
Anti-Rabbit	Biotin	Biogenex	HK326-UR		
Anti-Goat	AP	Sigma	A4187 – 1ML		
Anti-IgG	Biotin	S. Biotech	1030-08		
Anti-IgM	Biotin	S. Biotech	1020-08		
Streptavidin	AP	Biogenex	HK321-UK		
Streptavidin	PO	Biogenex	HK320-UK		

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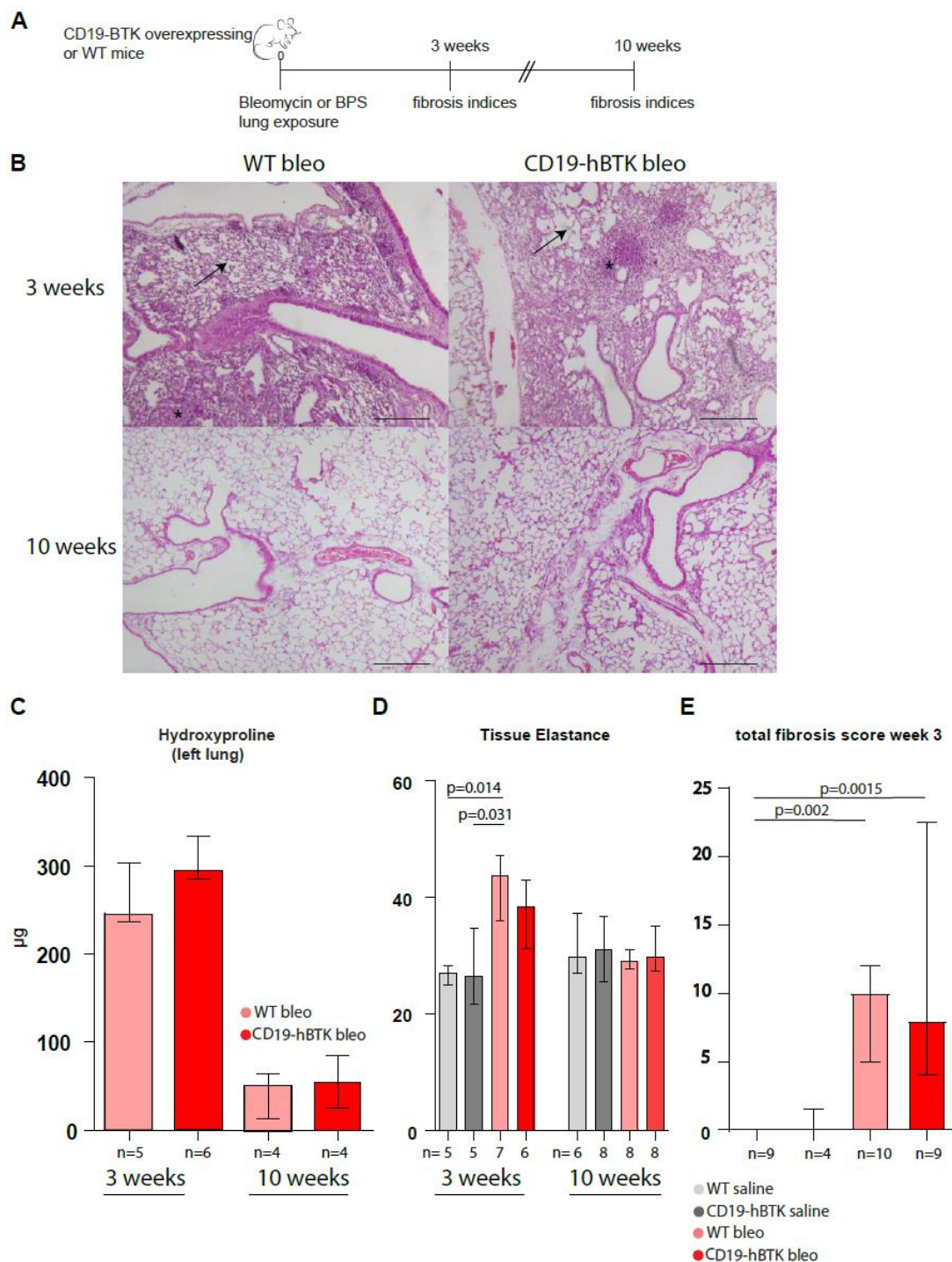
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180 **Online Supplementary Figure 1. Similar fibrosis indices in WT and CD19-hBTK mice upon bleomycin**
 181 **exposure.**



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183 (A) Mice were sacrificed 3 and 10 weeks after saline or bleomycin exposure and analyzed for fibrosis
 184 indices. (B) Representative hematoxylin/eosin (H&E) staining of cryo-sections of lung tissue of a WT or
 185 CD19-hBTK mouse 3 and 10 weeks after bleomycin exposure. Inflammatory exudate and obvious

186 damage to lung architecture (asterisk) and diffuse fibrous thickening of alveolar septa (arrow).
187 Resolution of fibrosis at 10 weeks. Magnification 40x. (C) Hydroxyproline content (μg per left lung) in
188 WT or CD19-hBTK mice 3 and 10 weeks post bleomycin exposure (D) Tissue elastance assessed with a
189 flexiVent FX system in WT or CD19-hBTK mice 21 days and 70 days post saline or bleomycin exposure.
190 (E) Total fibrosis score. A pathologist (blinded for treatment) scored the Ashcroft scale (grade 1-8) and
191 the percentage of lung involvement (grade 1-5; 1 = 0-10% to 6 = 75-100% of total lung involvement).
192 The total fibrosis score is the product of Ashcroft scale and lung involvement. The results (C-E) are
193 shown as median (IQR), p exact values were obtained following a Kruskal-Wallis test. Number of mice
194 used for each experiment are depicted below the graph.

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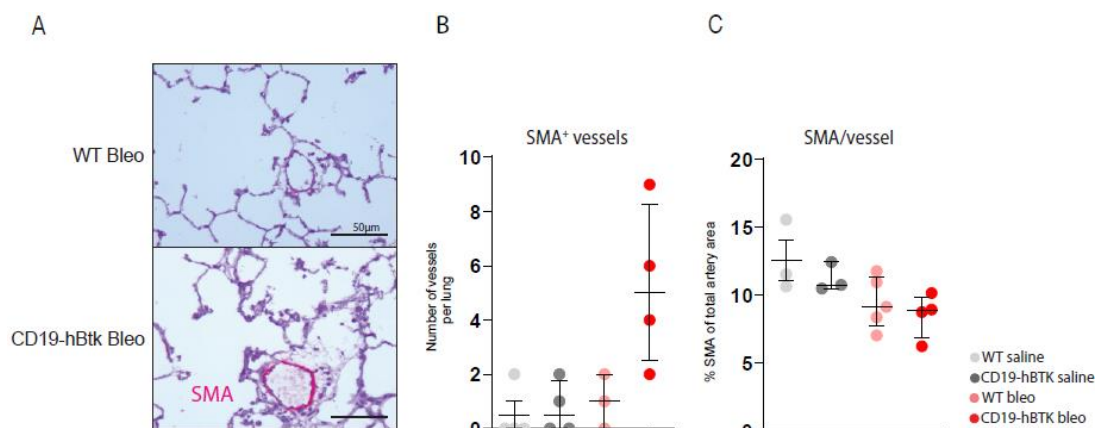
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212 **Online Supplementary Figure 2. α -SMA in WT or CD19-hBTK mice 10 weeks post bleomycin exposure.**

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215 (A) Representative hematoxylin/eosin and α -smooth muscle actin (α -SMA) staining of cryo-sections of
 216 the indicated lung tissue. (B) Number of SMA-positive vessels per lung. Vessels between 15 and 50 μ m
 217 external diameter and located in normal lung tissue were assessed. (C) Percentage α -SMA area to total
 218 artery area of the assessed vessels. The results are shown as median (IQR), p exact values were
 219 obtained following Kruskal-Wallis test. Dots represent individual mice.

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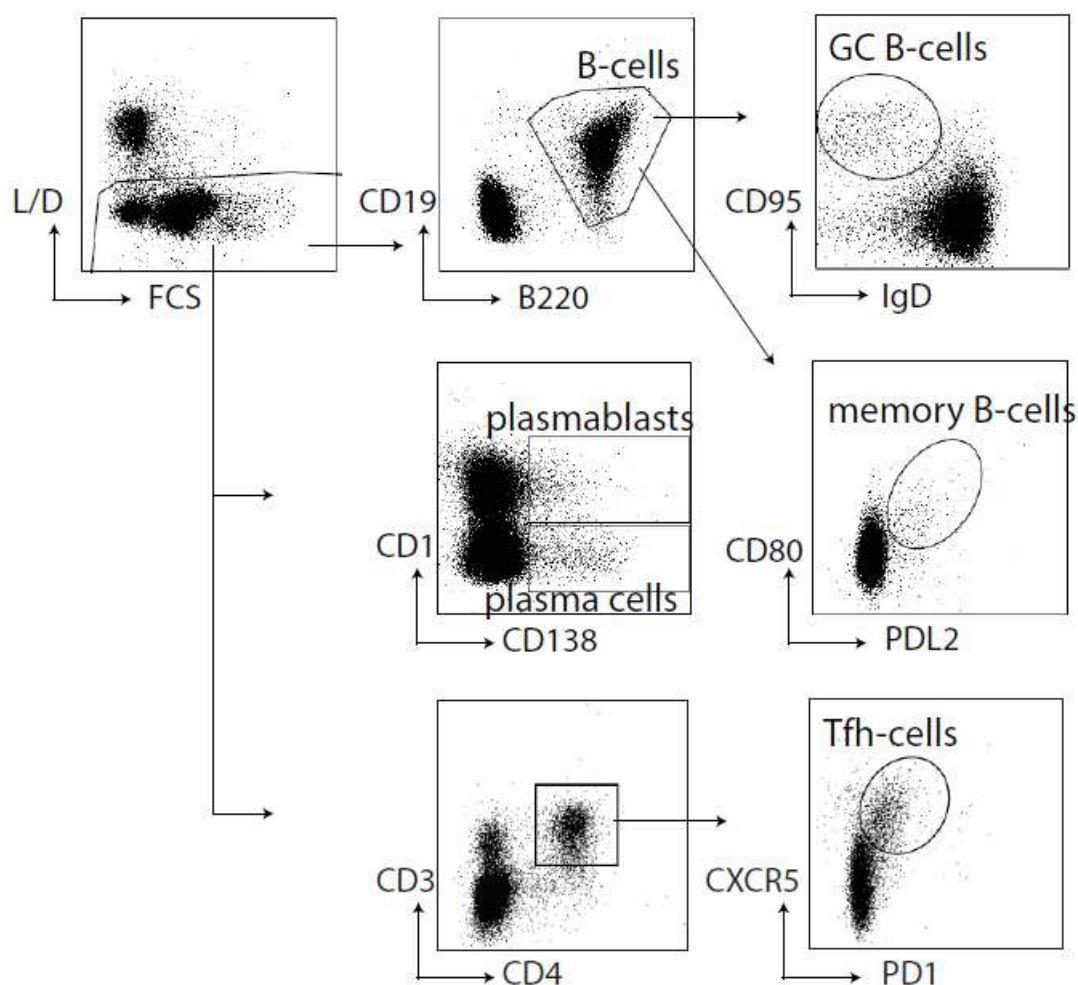
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232 **Online supplementary figure 3**

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234 Representative gating strategy for identification of B-cell subsets and follicular T helper (Tfh)-cells in
 235 mediastinal lymph nodes (MedLN) in mice, starting with live single cells. L/D, life-death marker; FCS,
 236 forward scatter. From the total CD19⁺B220⁺ B-cell population, memory B-cells
 237 (CD19⁺B220⁺CD80⁺PDL2⁺), germinal center (GC) B-cells (CD19⁺B220⁺IgD⁺CD95⁺), plasmablasts
 238 (CD19⁺CD138⁺) and plasma cells (CD19^{low}CD138⁺) were identified. From the CD3⁺CD4⁺ population, total
 239 numbers of Tfh-cells (CD3⁺CD4⁺CXCR5⁺PD1⁺) were identified.

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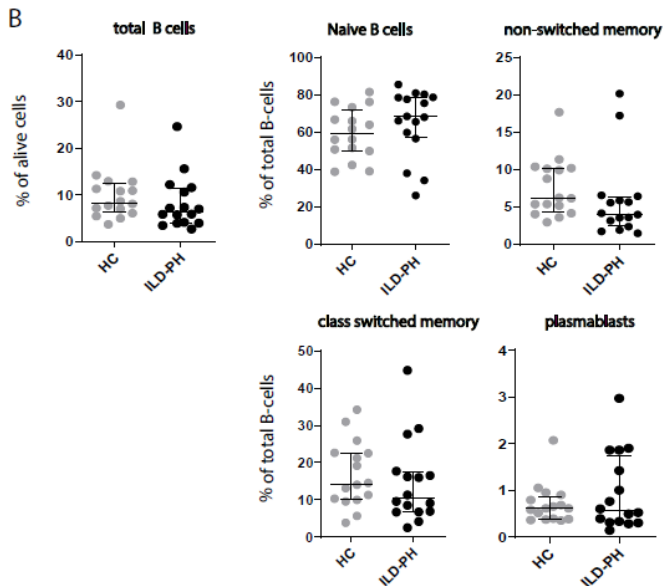
247 **Online Supplementary Figure 4: B-cell subsets and BTK expression in ILD-PH**

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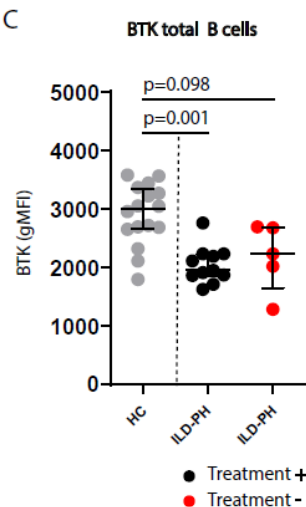
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	gender (male)	age (years)	mPAP (mmHg) ^A	Immunomodulating therapies ^B	Anti fibrotics ^C
HC (N=16)	10 (63%)	59 (54-64.5)	-	-	-
ILD-PH (N=17) ^D	5 (29%)	63 (57.5-76)	35 (27-40)	5 (29%)	7 (41%)

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C



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250 (A) Table showing the characteristics of patients with interstitial lung disease-related PH (ILD-PH) and
 251 healthy subject (HC). mPAP; mean pulmonary artery pressure. ^A In 6 patients only echocardiographic
 252 measurements available, showing estimated RVSP> 35mmHg and signs of RV dysfunction and no signs
 253 of left heart disease or left sided heart failure. ^B Four patients used prednisone >10mg/day and one
 254 patient was on azathioprine. ^C Nintedanib (n=3) or pirfenidone (n=4). ^D Ten patients with IPF, 3 patients
 255 with non-specific interstitial pneumonia, 1 patient with respiratory bronchiolitis interstitial lung
 256 disease, 1 patient with extrinsic allergic alveolitis, 1 patient with combined pulmonary fibrosis and
 257 emphysema, and 1 patient with interstitial pneumonia with autoimmune features. Continuous
 258 variables are presented as median and IQR in parentheses and categorical variable as count and
 259 percentages in parentheses.

260 (B) Proportions of circulating total B-cells and B-cell subpopulations (naïve B-cells (CD19⁺IgD⁺CD27⁻),
 261 non-switched memory B-cells (CD19⁺IgD⁺CD27⁺), and class switched memory B-cells (CD19⁺CD27⁺IgD⁻
 262 IgM⁺), and plasmablasts (CD19⁺CD38^{hi}CD27⁺)) in HC and patients with ILD-PH. (C) Quantification of BTK
 263 protein expression levels, shown as gMFI values of intracellular flow cytometry analysis of total B cells

264 in HCs and ILD-PH patients receiving immunomodulatory or anti-fibrotic treatment (*black dots*) or no
265 treatment (*red dots*). p exact values were obtained by a Mann-Whitney U test or Kruskal-Wallis test
266 (>2 groups). Dots represent individual values in patients.

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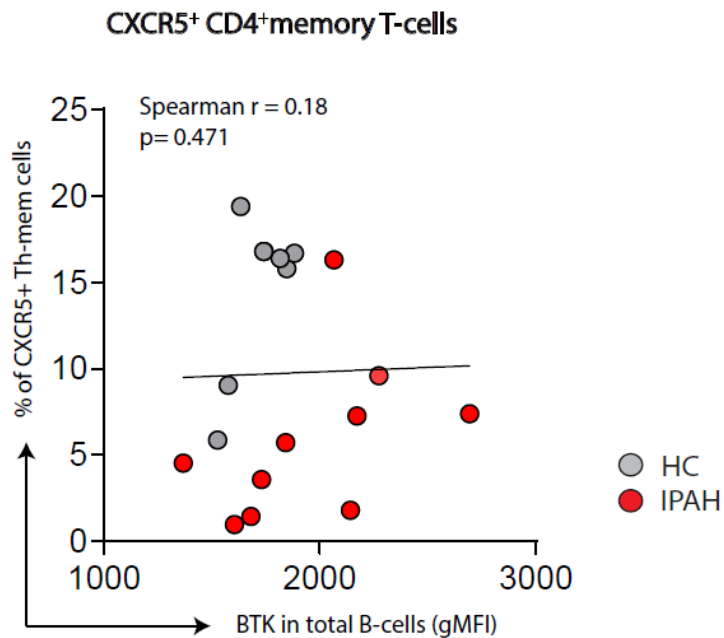
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299 **Online Supplementary Figure 5: No correlation of cTfh cells and BTK protein in total B-cells**

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302 No correlation of circulating follicular T helper (cTfh) cells (CD4⁺CD45RA⁻FoxP3^{low}CXCR5⁺) and BTK
303 protein in total B cells in HC and patients with IPAH. Correlation coefficient was calculated using
304 Spearman's rank method.

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