Supporting Information

Quantification and Role of Innate Lymphoid Cell Subsets in COPD

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Supporting Information includes:

(1) Supplementary tables:

       Supplementary table 1

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(2) Supplementary figures:

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Supplementary table 1

	Never-smoking controls	Smoking (ex + current) controls	COPD	
	Median (IQR)	Median (IQR)	Median (IQR)	*P*
n	3	6	15	
Pack years	0 (0-0)	15.5 (3-28.75)	40 (34.5-50)	0.0019
Age (years)	76 (67-76.5)	59.5 (56.25-65)	65 (58-69)	0.3424
% ILC1	19.9 (17.8-20.13)	40 (35.28-52.83)	63.5 (40.8-71.35)	0.0323
% ILC2	1.97 (1.26-7.94)	3.38 (1.97-7.91)	3.35 (1.65-4.87)	0.8578
% NKp44+ ILC3	3.23 (2.56-3.78)	3.84 (2.19-5.84)	4.02 (1.52-7.91)	0.9144
% NKp44- ILC3	39.3 (28-57.25)	34.15 (29.2-41.58)	21.1 (11.9- 29.95)	0.3009

Supplemental table 1 : ILC percentages in human lung tissue.
The % of ILC subsets (median and interquartile range, IQR) within the CD45^{+}, Lin^{-}CD161^{+} CD127^{+} population are shown for never-smoking controls, smoking controls (ex+current) and COPD. Variables were compared with a Kruskal-Wallis test.

Supplementary table 2

	Unadjusted β (se), *P*	Model 1 β (se), *P*	Model 2 β (se), *P*
CAT	1.22 (0.56), $P = 0.04$	0.93 (0.51), $P = 0.08$	0.84 (0.56), $P = 0.08$
CAT4	6.56 (2.12), $P = 0.01$	5.14 (2.03), $P = 0.02$	3.95 (1.96), $P = 0.06$
FEV1%	-0.36 (0.18), $P = 0.06$	-0.18 (0.22), $P = 0.41$	0.05 (0.21), $P = 0.81$
FEV1/FVC	-0.71 (0.41), $P = 0.10$	-0.18 (0.50), $P = 0.72$	-0.02 (0.46), $P = 0.97$
DLCO	-0.38 (0.23), $P = 0.11$	-0.19 (0.23), $P = 0.43$	-0.17 (0.19), $P = 0.39$

Supplemental table 2 : Linear regression analyses for the association between CAT scores or lung function and % of ILC1 in human lung tissue.
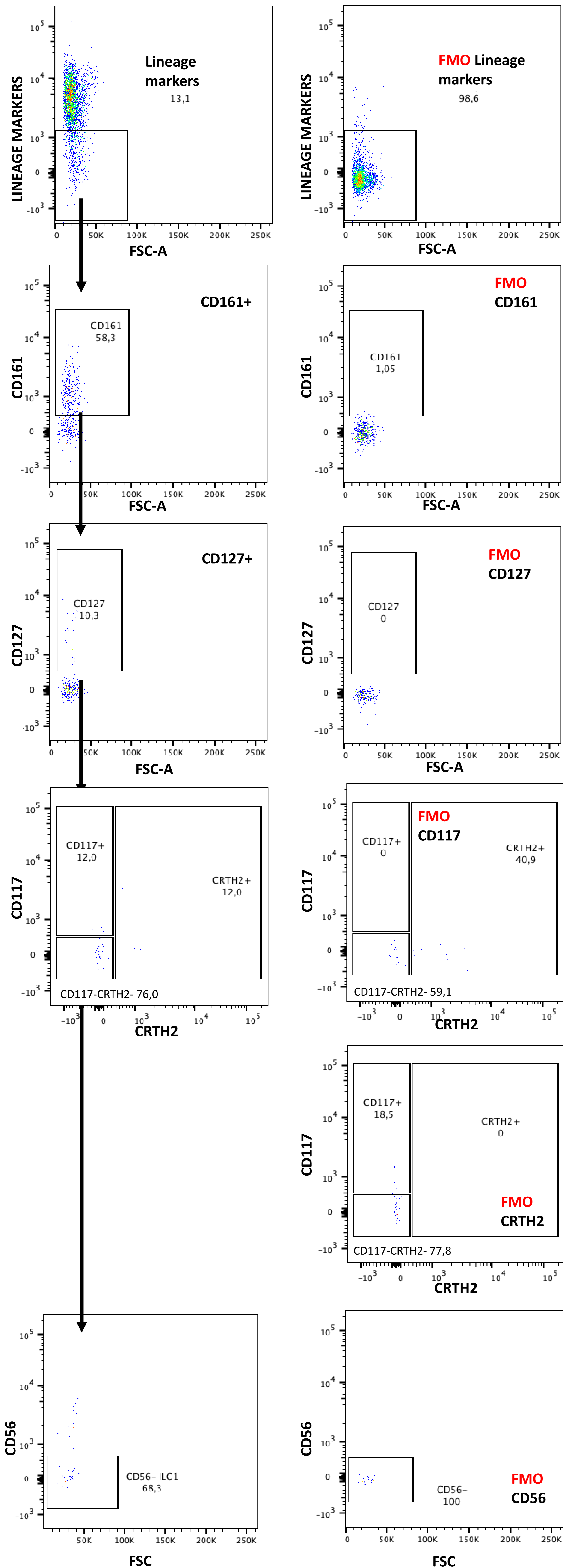Regression coefients (β) and standard errors (se) for the association between independent variables and ILC1 are given.
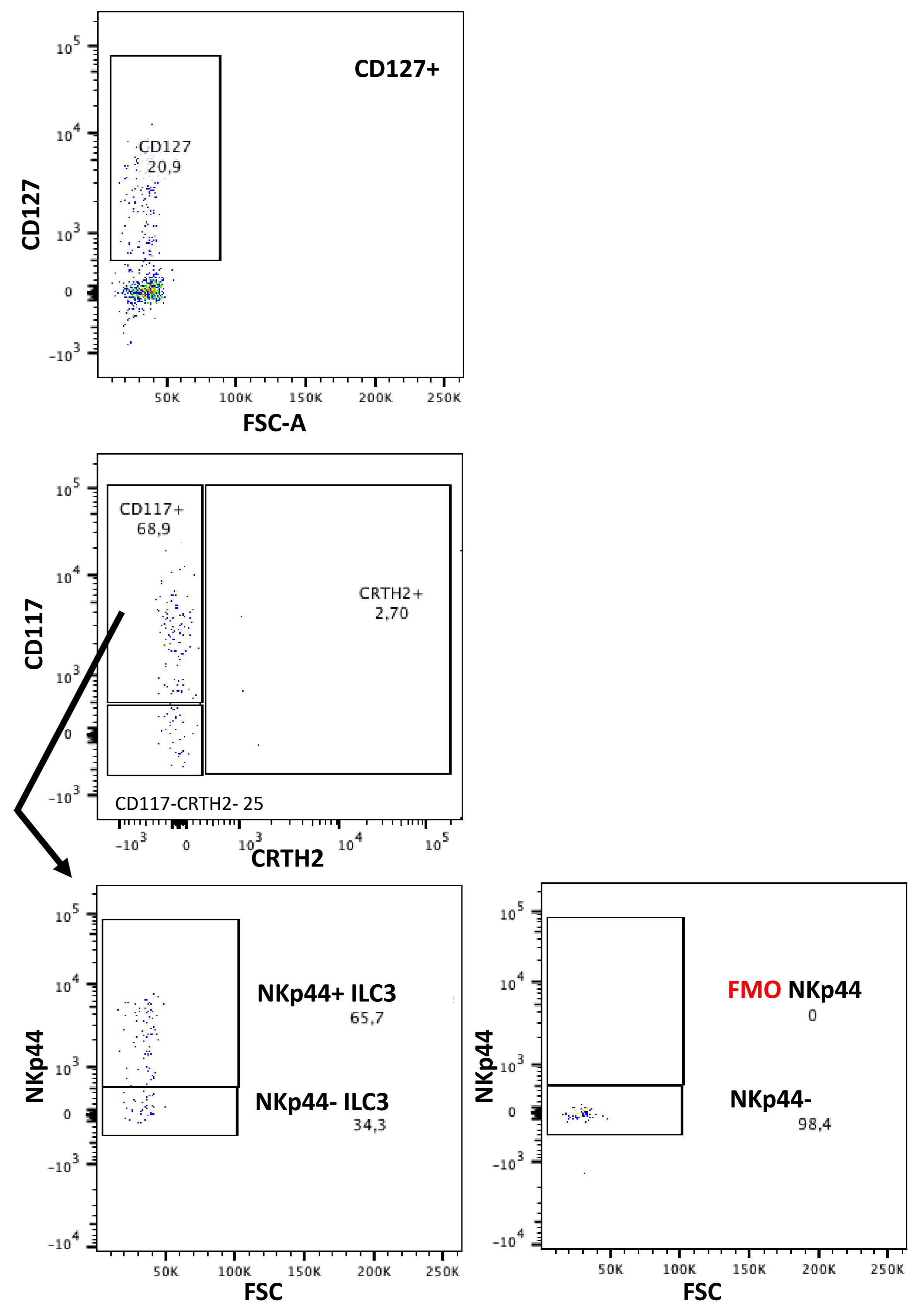Model 1 is adjusted for ever smoking
Model 2 is adjusted for age, sex and ever smoking

Supplementary figure 1

**a)** Within CD45+, live cells, lymphogate:

b) Within CD45+, live, lymphogate, lin-, CD161+:

Supplementary figure 1: FMOs and gating of Lineage markers, CD161 and CD127 and other markers to identify the human ILCs with the surface staining. For flow cytometric analysis, ILC were surface stained and gated as live, CD45^{+} lymphocytes that are Lin[-](CD3, CD19, CD11c, CD11b, CD1a, CD14, CD34, CD123, TCRαβ, TCRγδ, BDCA2, CD235A and FcεR1) CD161^{+} CD127^{+}.
Full staining (left) and FMOs (right) for subject X **(a)**, Full staining (left) and FMO for Nkp44 (right) for subject Y **(b)**

Supplementary figure 2

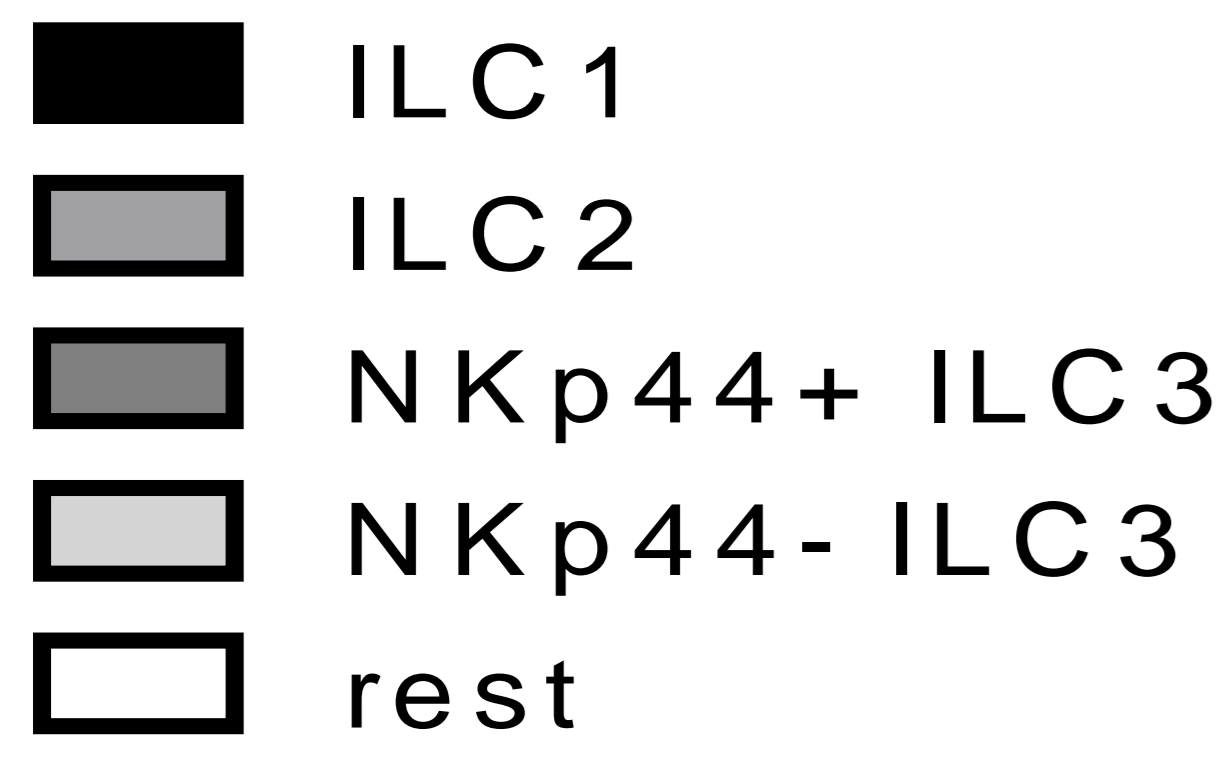**(a)** Within CD45+, live cells, lymphogate: **(b)**
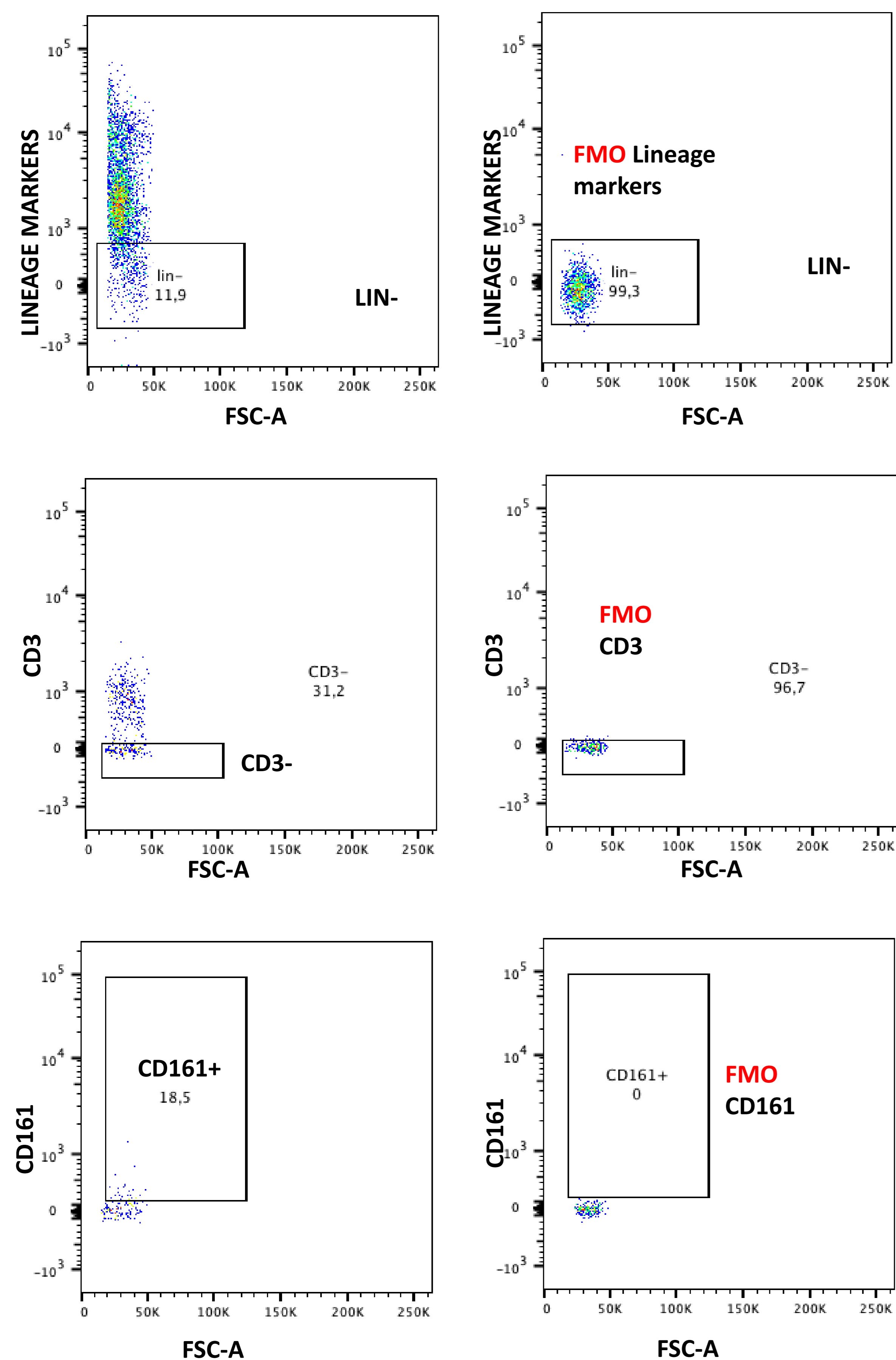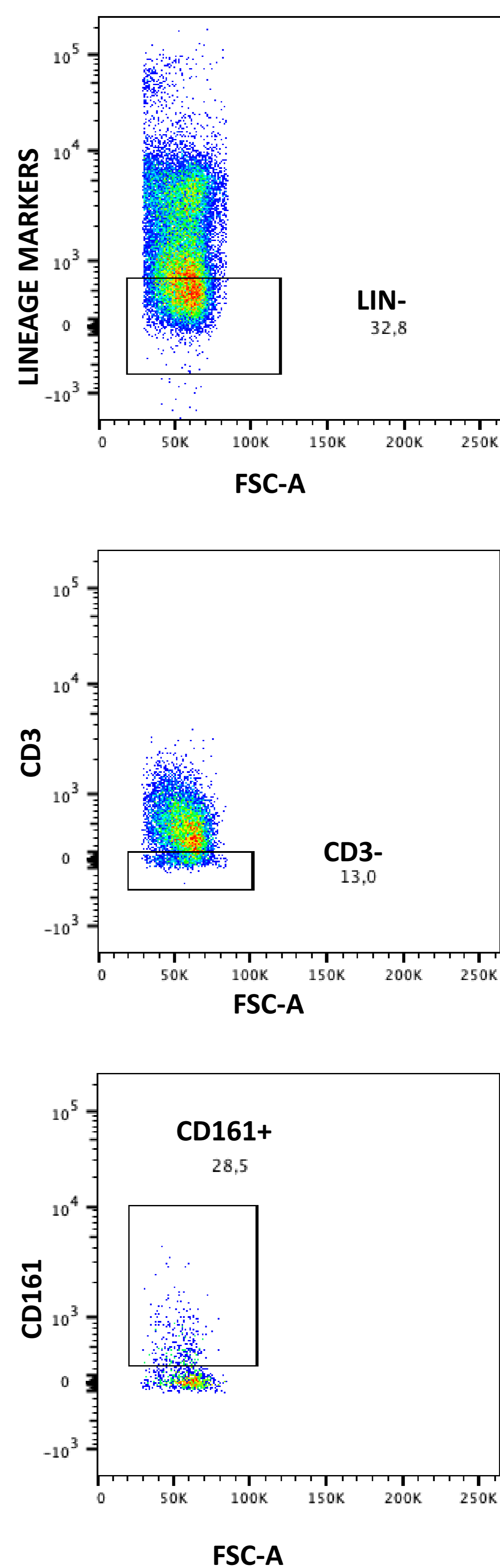
Supplementary figure 2: FMOs and gating of Lineage markers, CD3 and CD161 for the human ILCs for the cytokine staining.

For flow cytometric analysis of ILC that were labeled following 4h of stimulation (cytokine detection): ILC were gated as live, CD45+ lymphocytes that were Lin-(CD19, CD11c, CD11b, CD1a, CD14, CD34, CD123, TCRαβ, TCRγδ, BDCA2, CD235A and FcεR1) CD3-CD161+.

Full staining (left) and FMOs (right) for lineage markers, CD3 and CD161 for subject X **(a)**, Full staining for subject Y **(b)**

Supplementary figure 3

(a)

Legend:
- ■ ILC1
- ▦ ILC2
- ▨ NKp44+ ILC3
- ▤ NKp44- ILC3
- □ rest

Controls

COPD GOLD I

COPD GOLD II

COPD GOLD III

	Never-Smoker	Ex-Smoker	Current smoker

(b)

COPD n=15 control n=9

R=0.4674
P=0.0213

ILC1 (%) vs Pack years
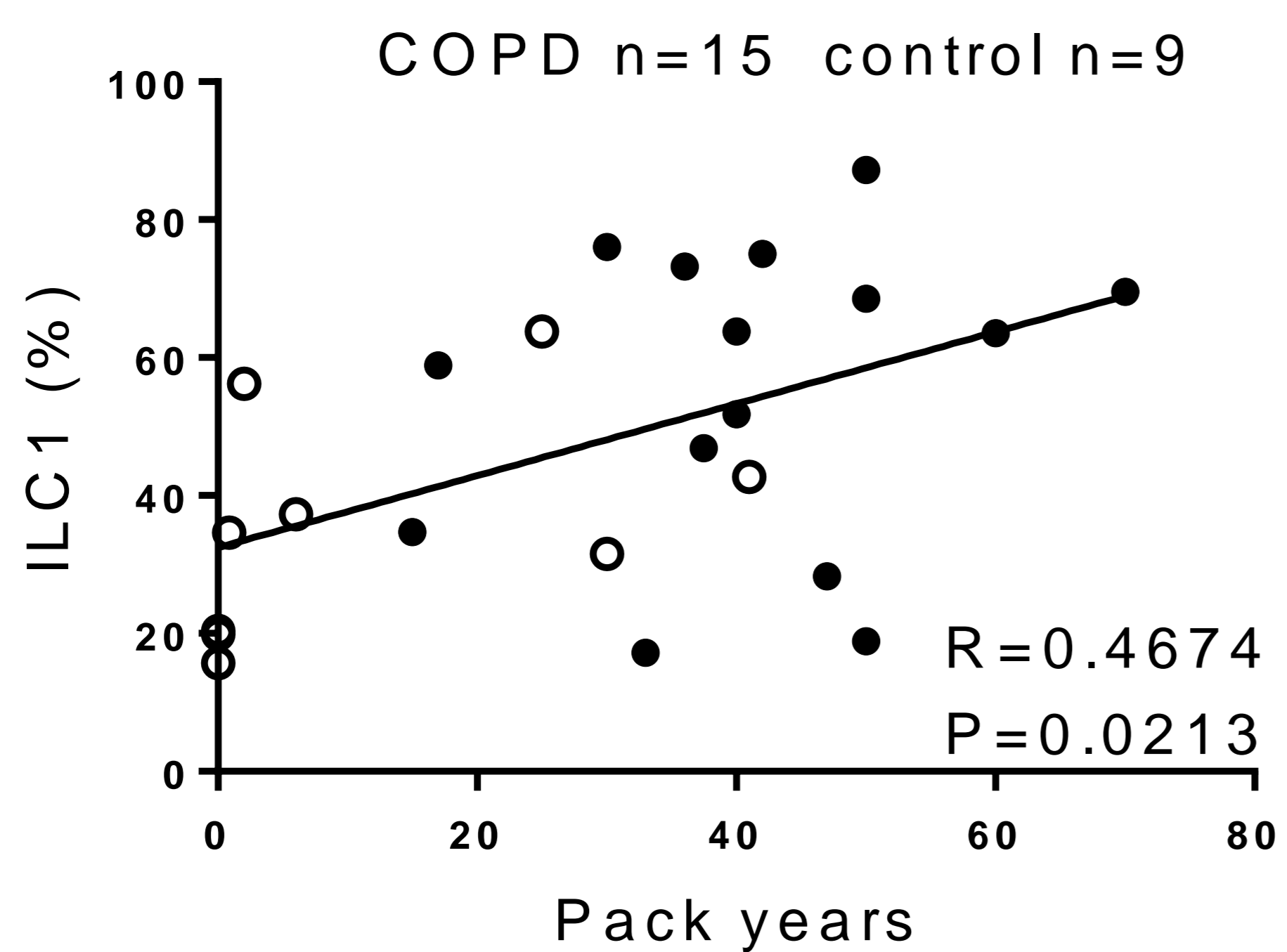
Supplementary figure 3: Distribution of ILC subsets smd correlation with pack years in human lung tissue. Distribution of ILC subsets in all included subjects individually, grouped in never-smoking controls, smoking (ex+current) controls and COPD **(a)** and Spearman correlation between % of ILC1 and pack years in controls (clear dots) and COPD patients (black dots) **(b)**
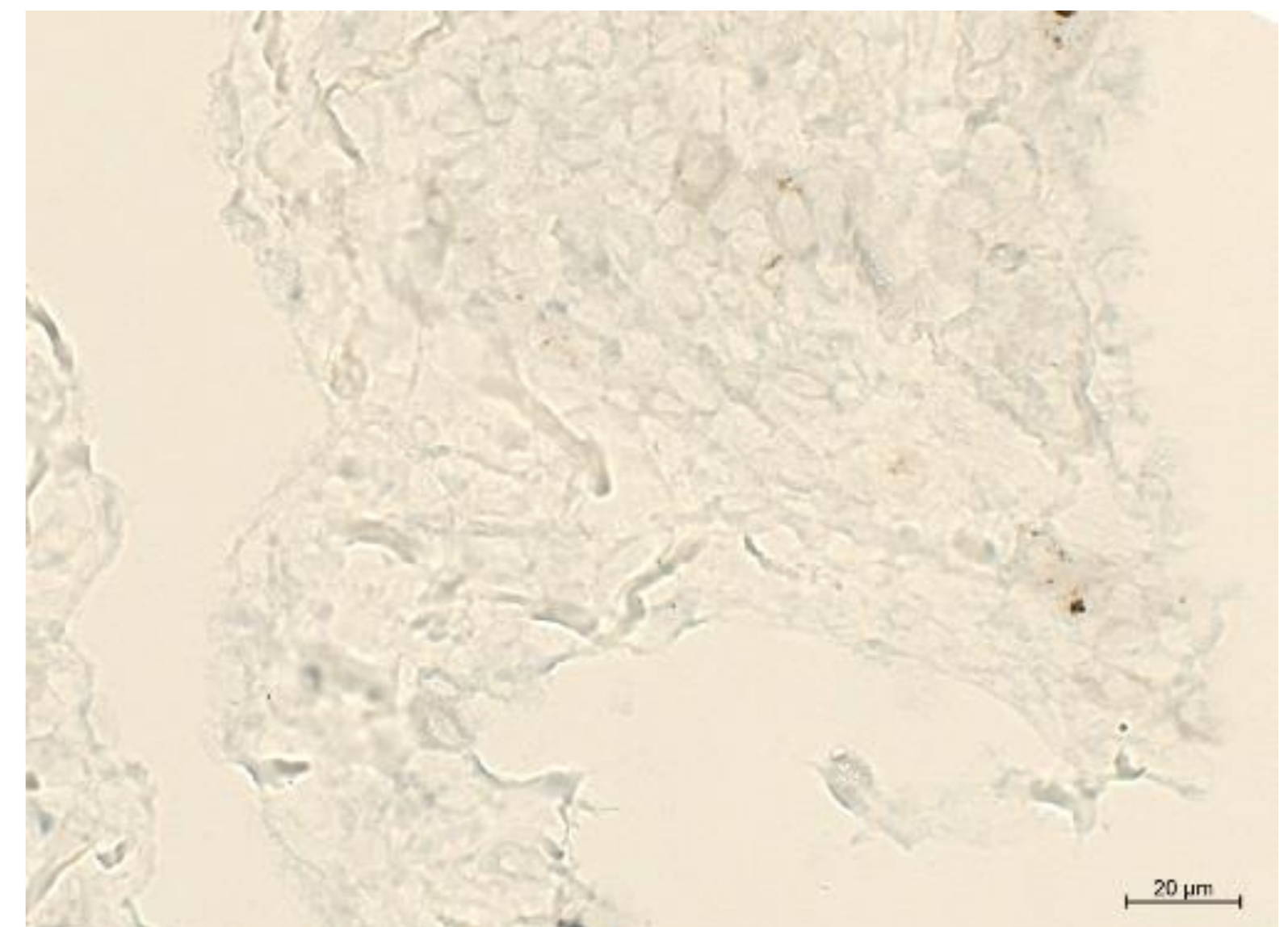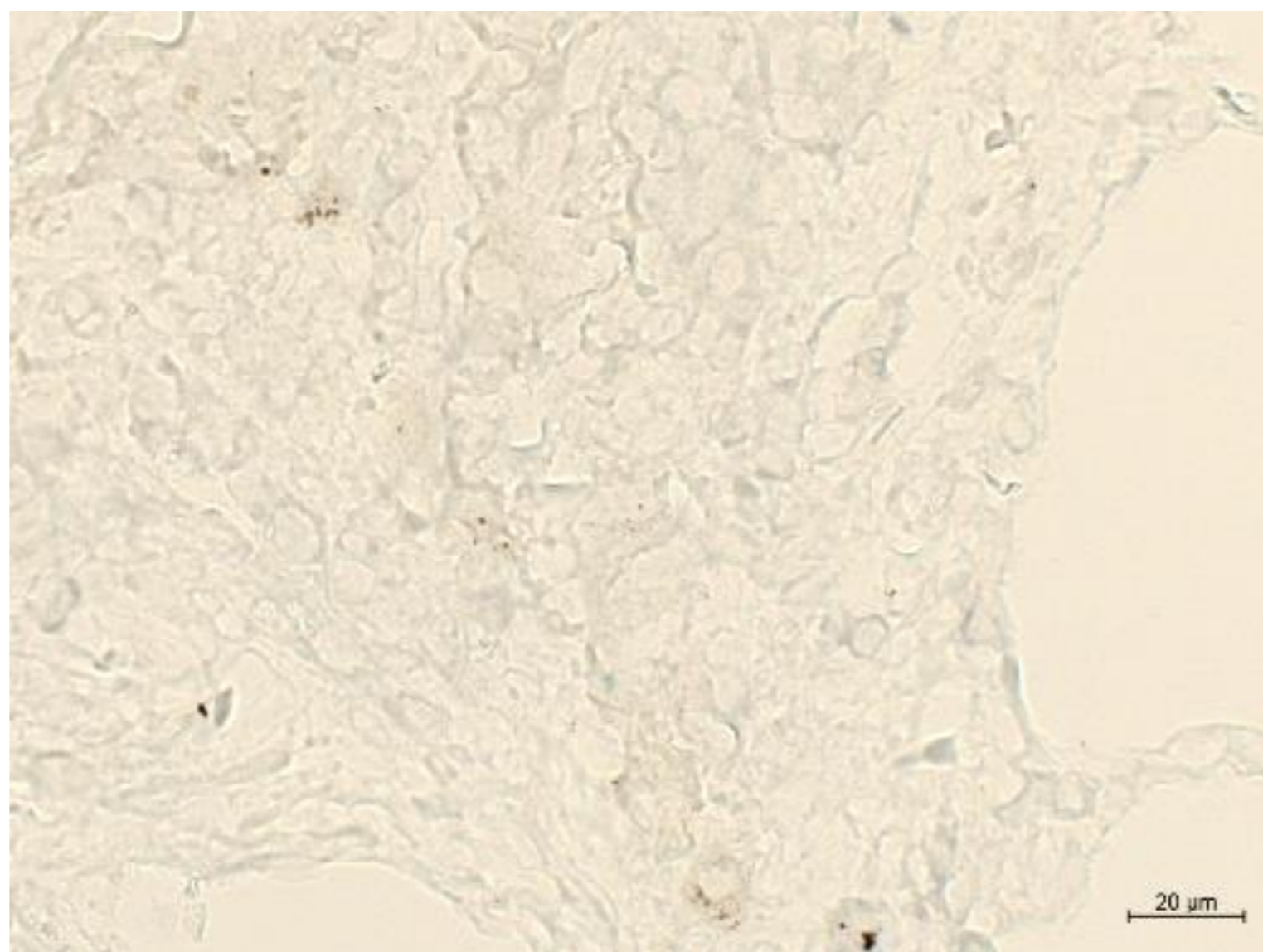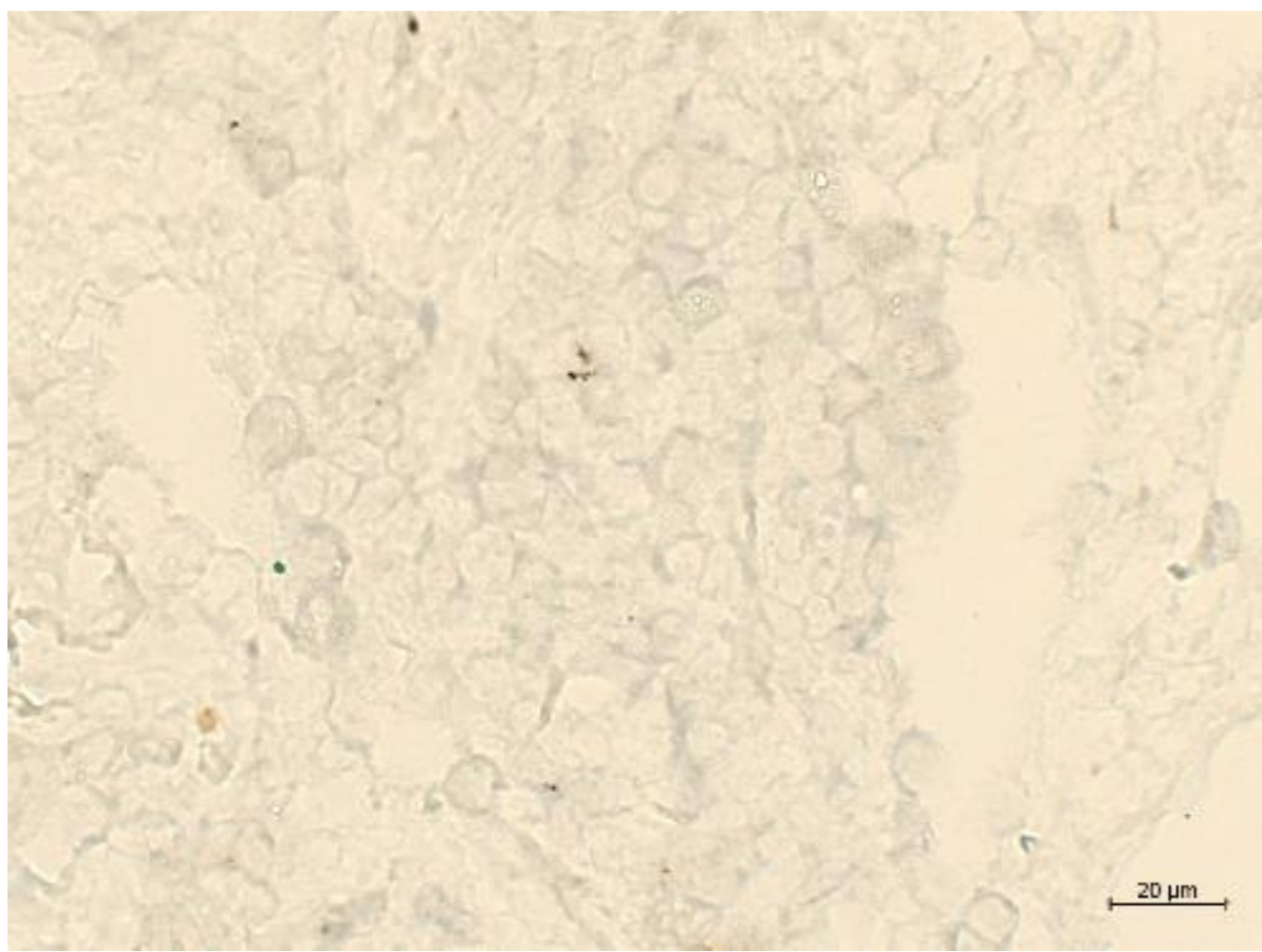
Supplementary figure 4

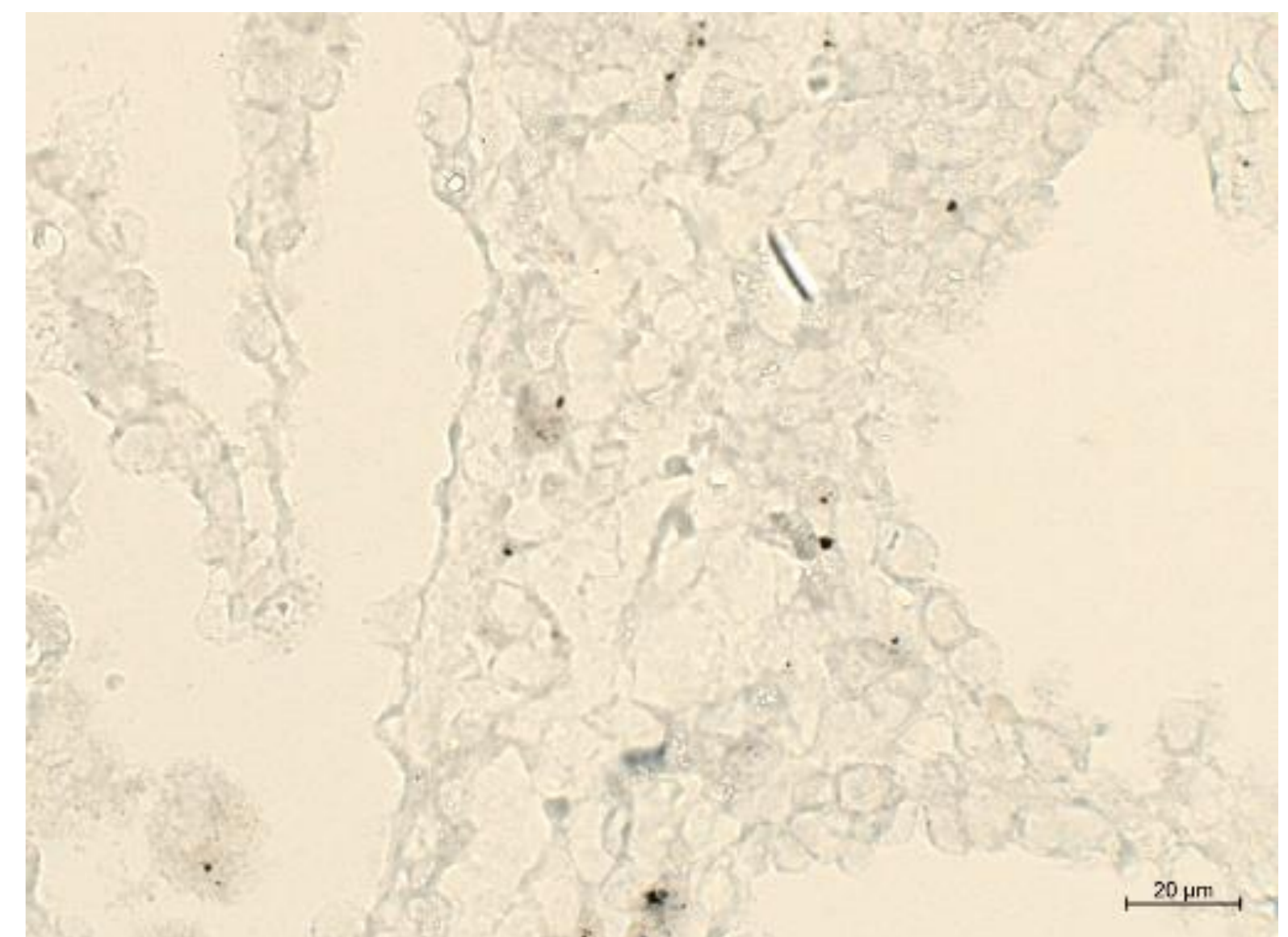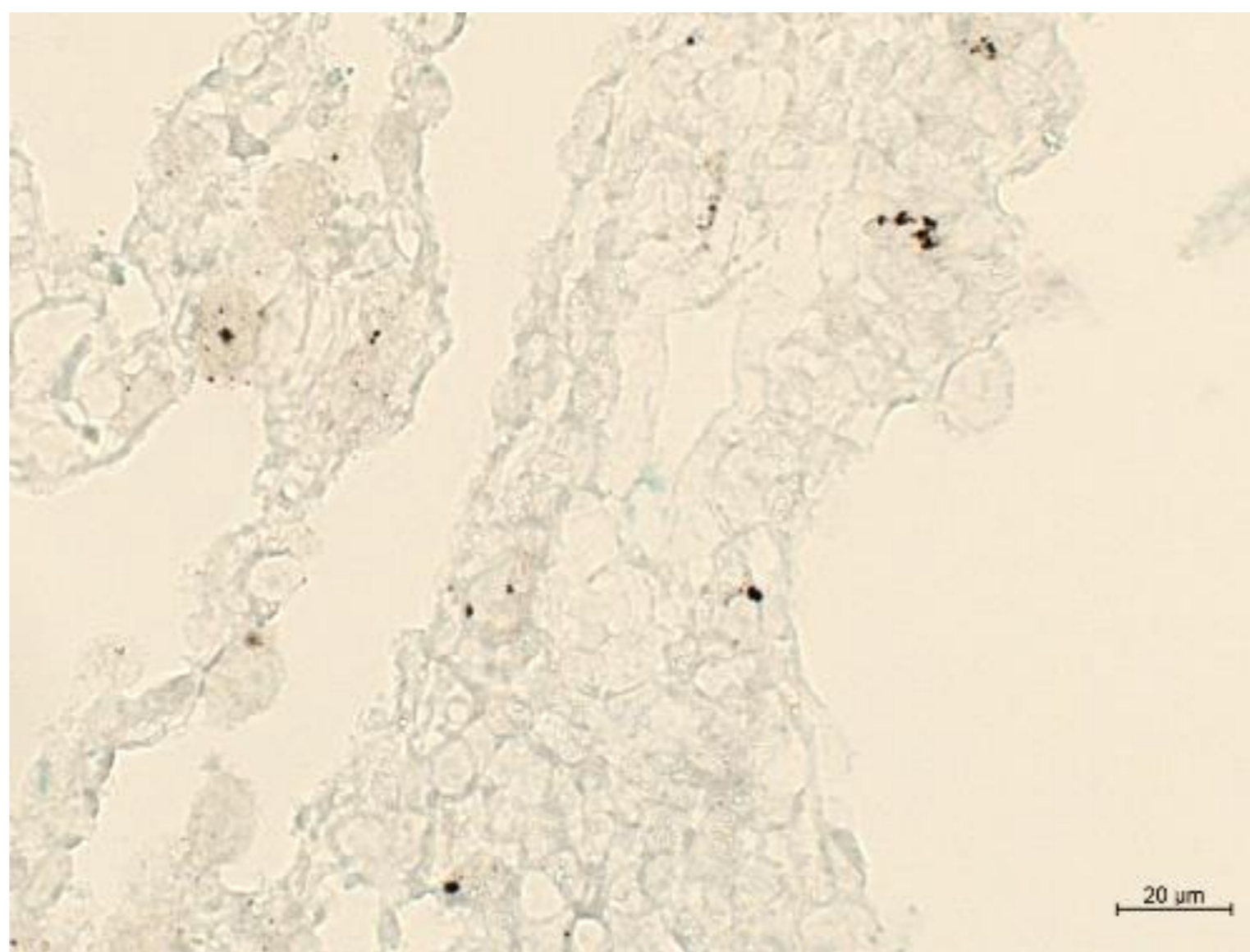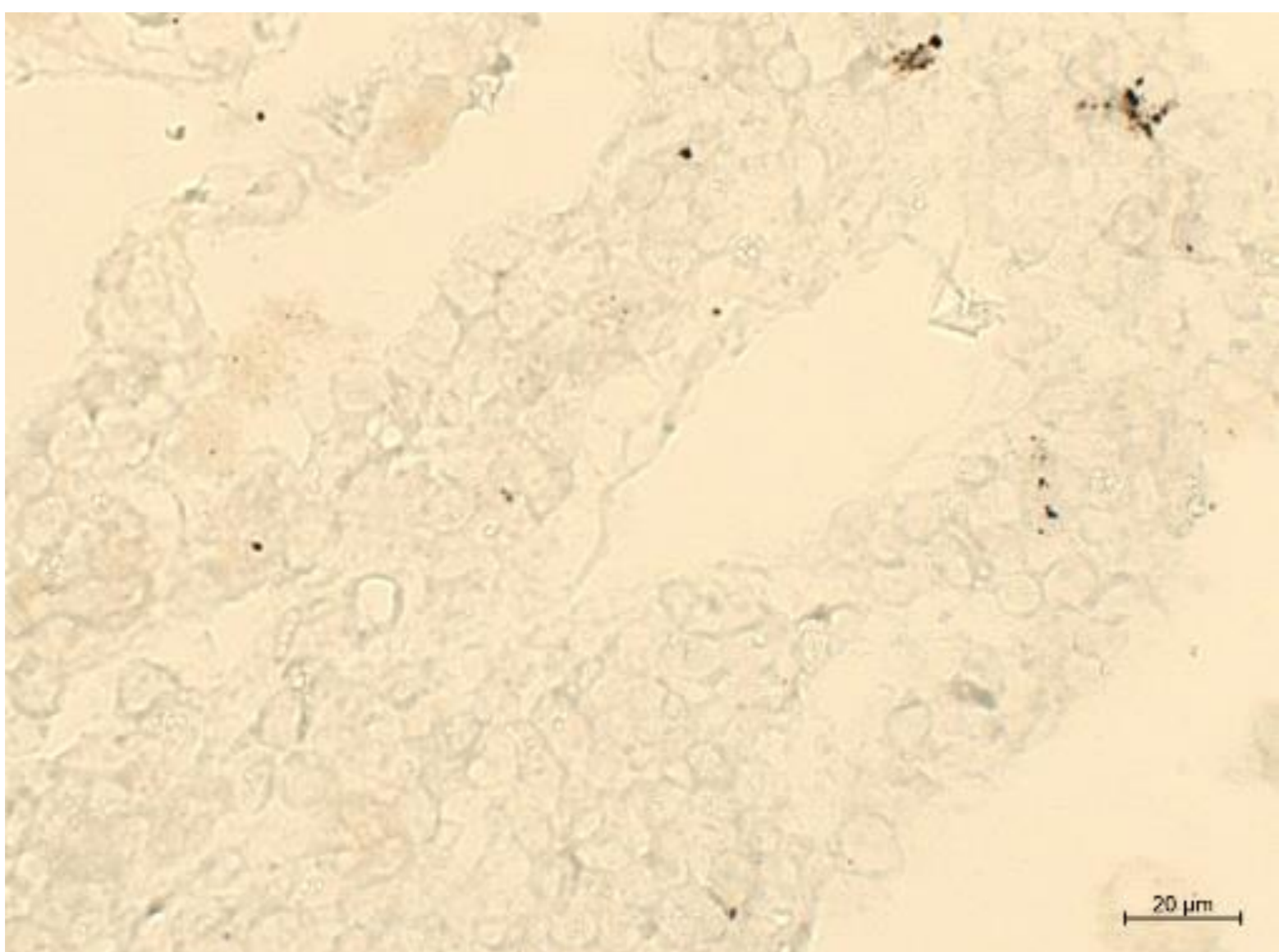|	**(a) ISO T-BET/CD3/cocktail**	**(b) ISO GATA3/CD3/cocktail**	**(c) ISO RORγt/CD3/cocktail**

Never-smoker
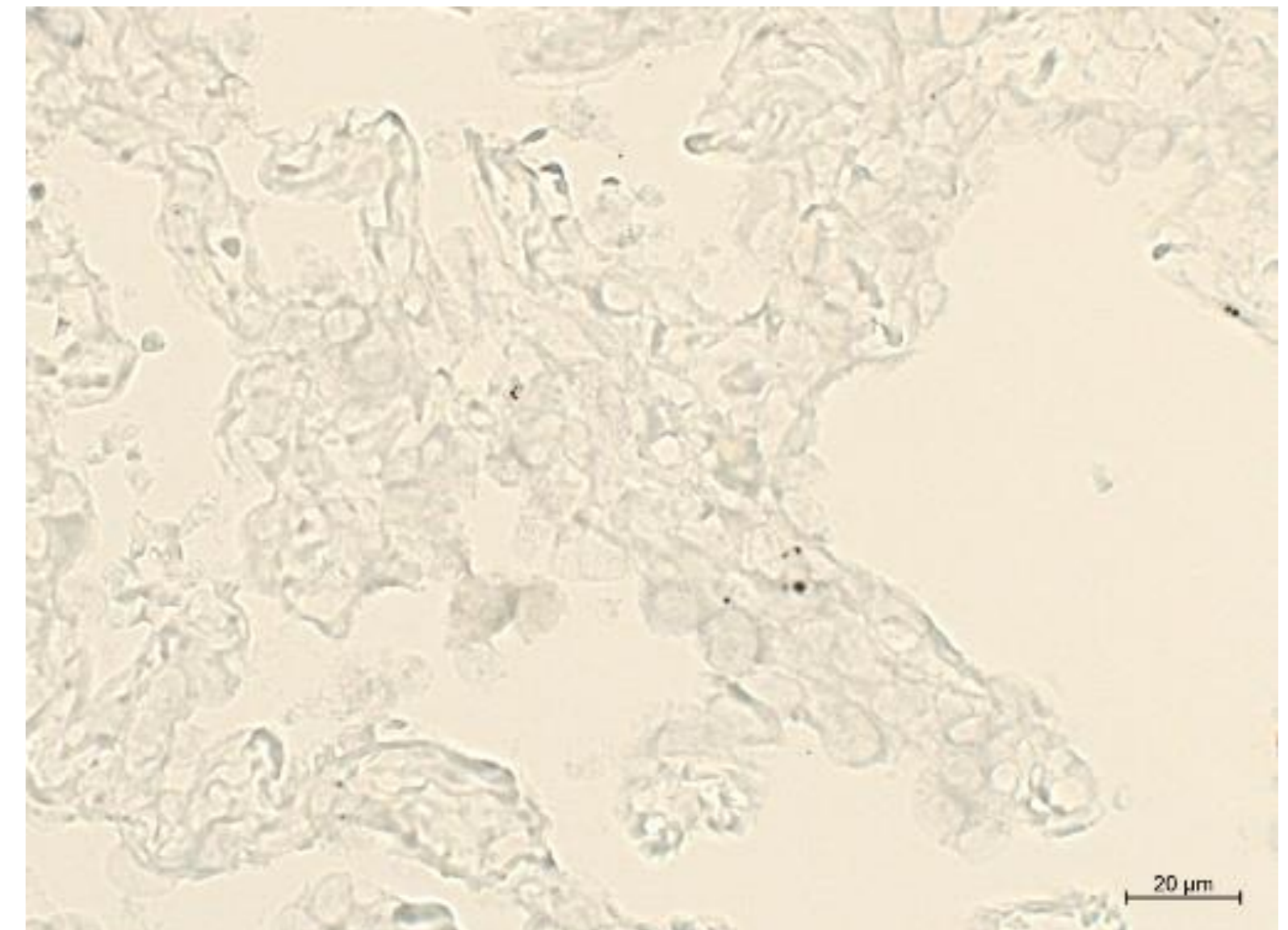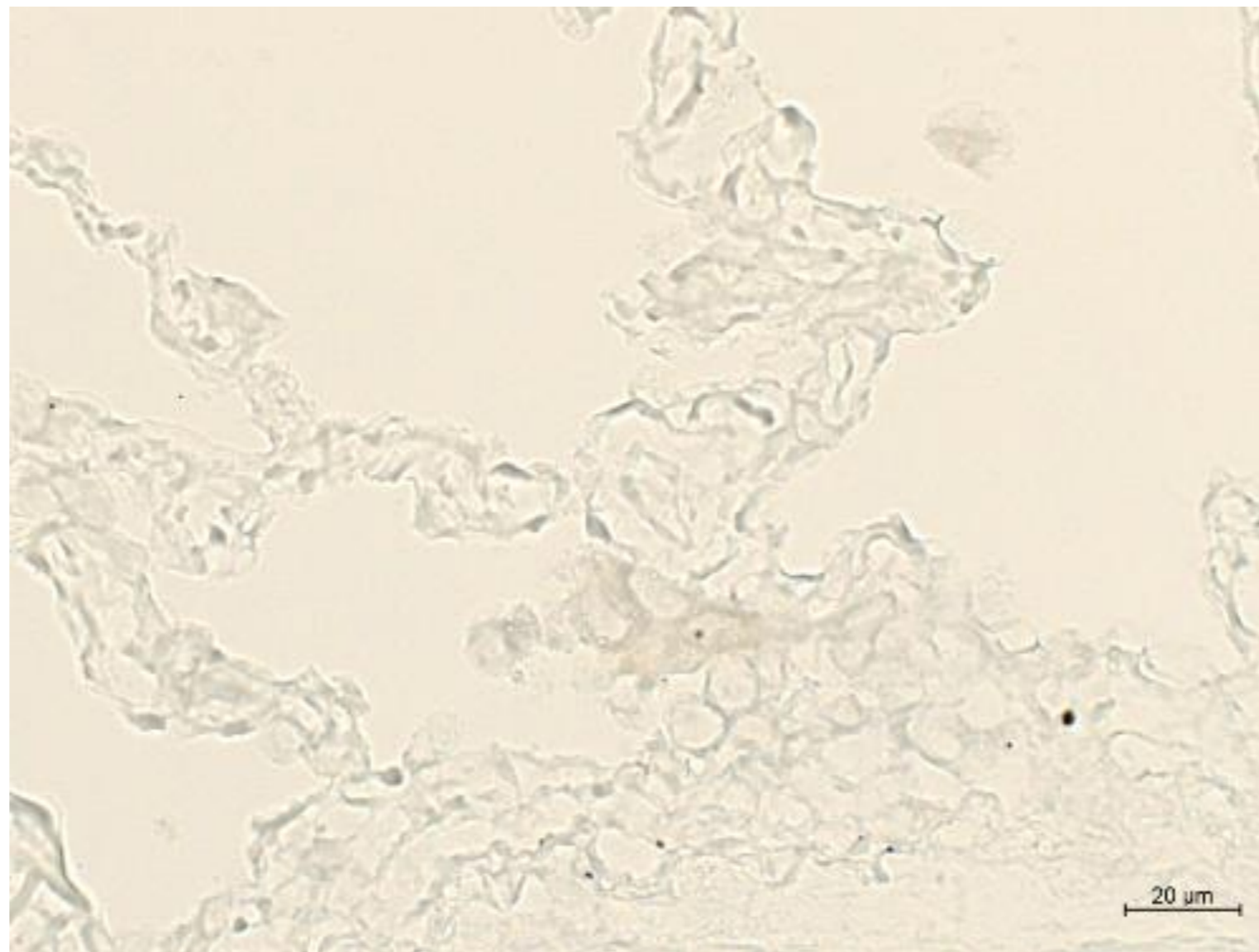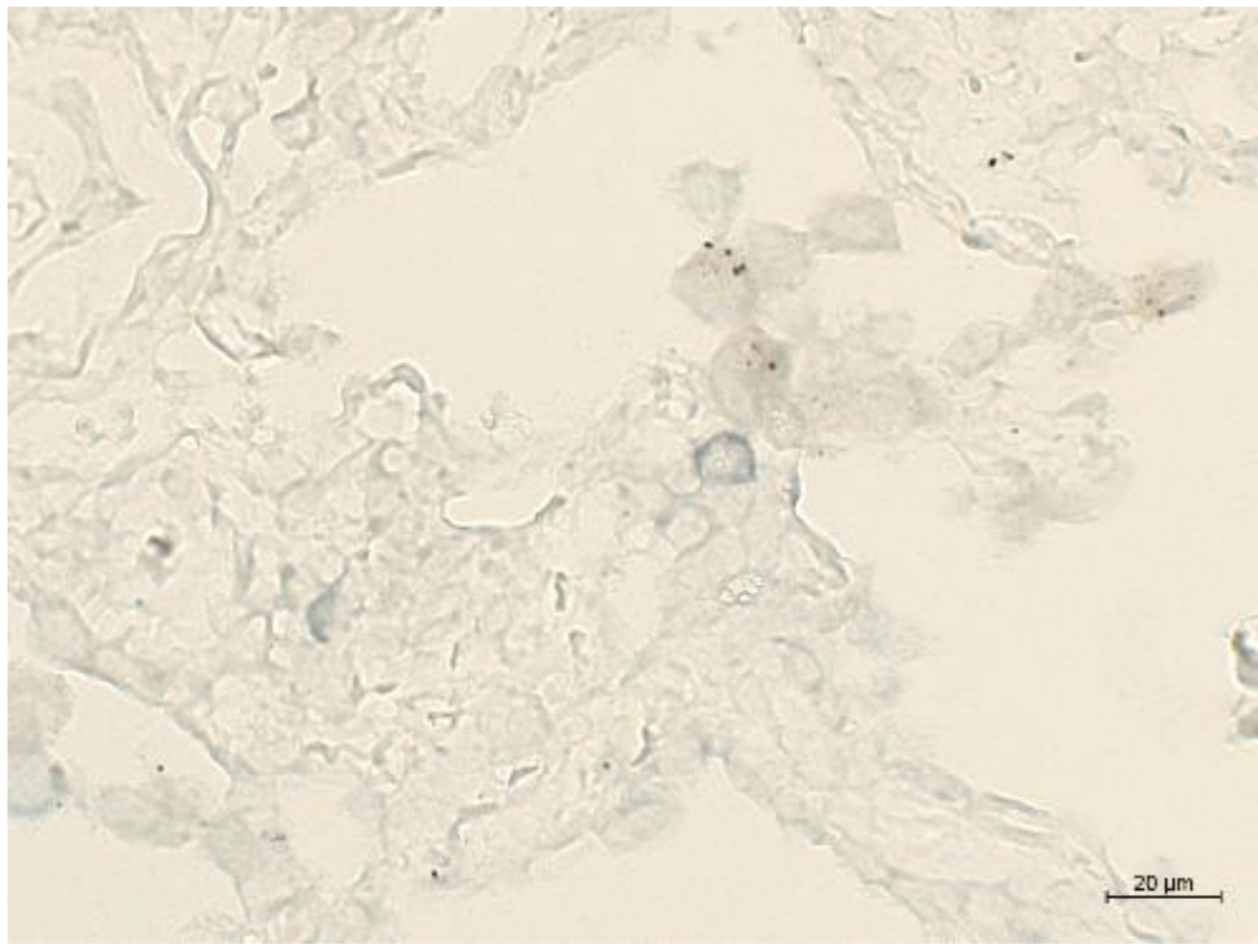
Smoker

COPD GOLD II (smoker)

Supplementary figure 4 : Isotype controls for immunohistochemical detection of pulmonary ILC and Th cells in COPD and control subjects: Isotype-matched control antibodies for: CD20, CD56, CD68, mast cell tryptase (exclusion cocktail), T-BET and CD3 **(a)** exclusion cocktail, GATA3 and CD3 **(b)** exclusion cocktail, RORγt and CD3 **(c)**. Scale bars: 20 µm

Supplementary figure 5
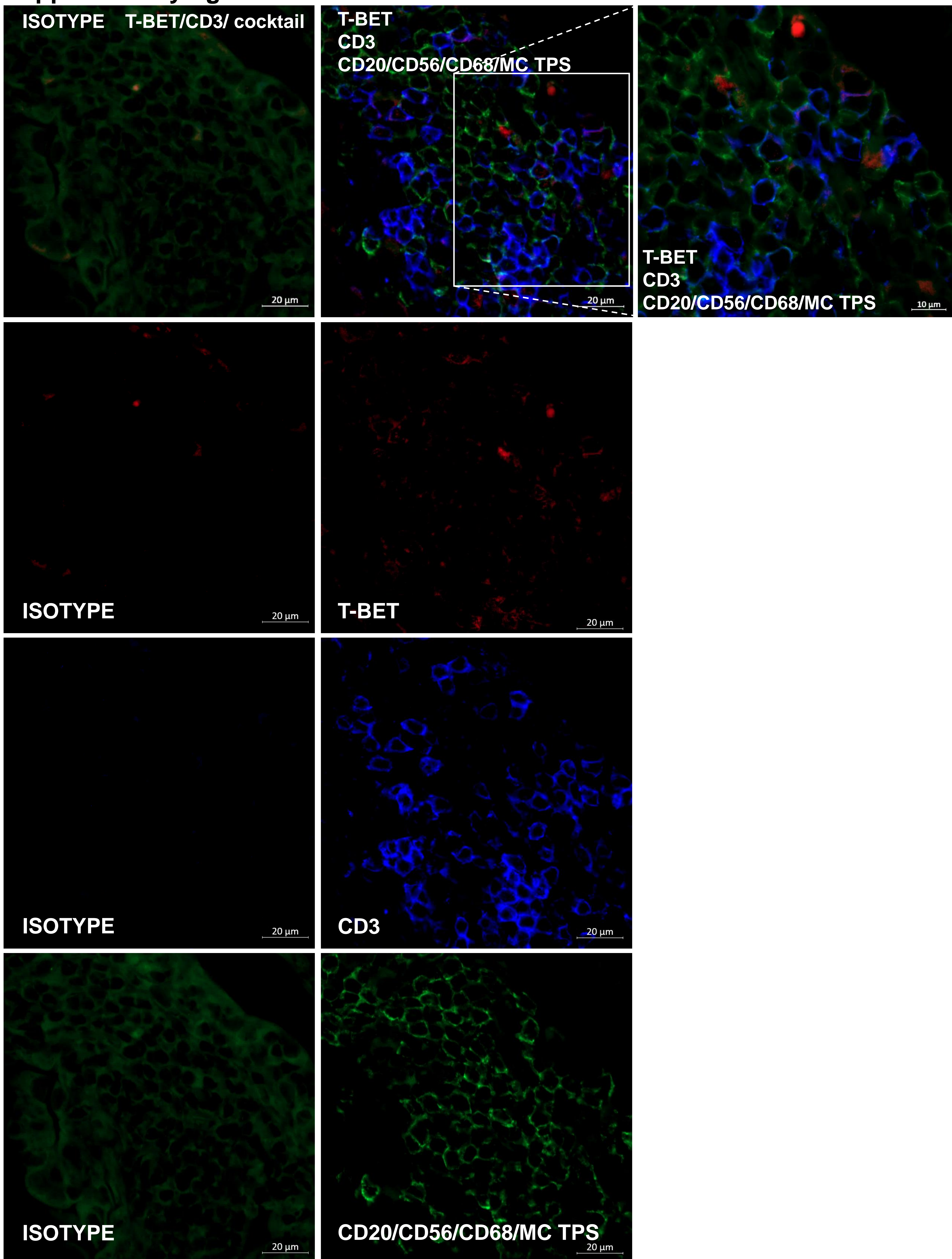
Supplementary figure 5: Isotype controls for immunofluorescent detection of pulmonary ILC and Th cells in COPD subject:
left panels: Isotype-matched control antibodies for: T-BET, CD3 and the exclusion cocktail (CD20, CD56, CD68, mast cell tryptase), **middle and right panel**: staining for T-BET, CD3 and the exclusion cocktail. Rare staining artefacts and autofluoresence in isotype controls can clearly be distinguished from positive staining in the antibody stainings.
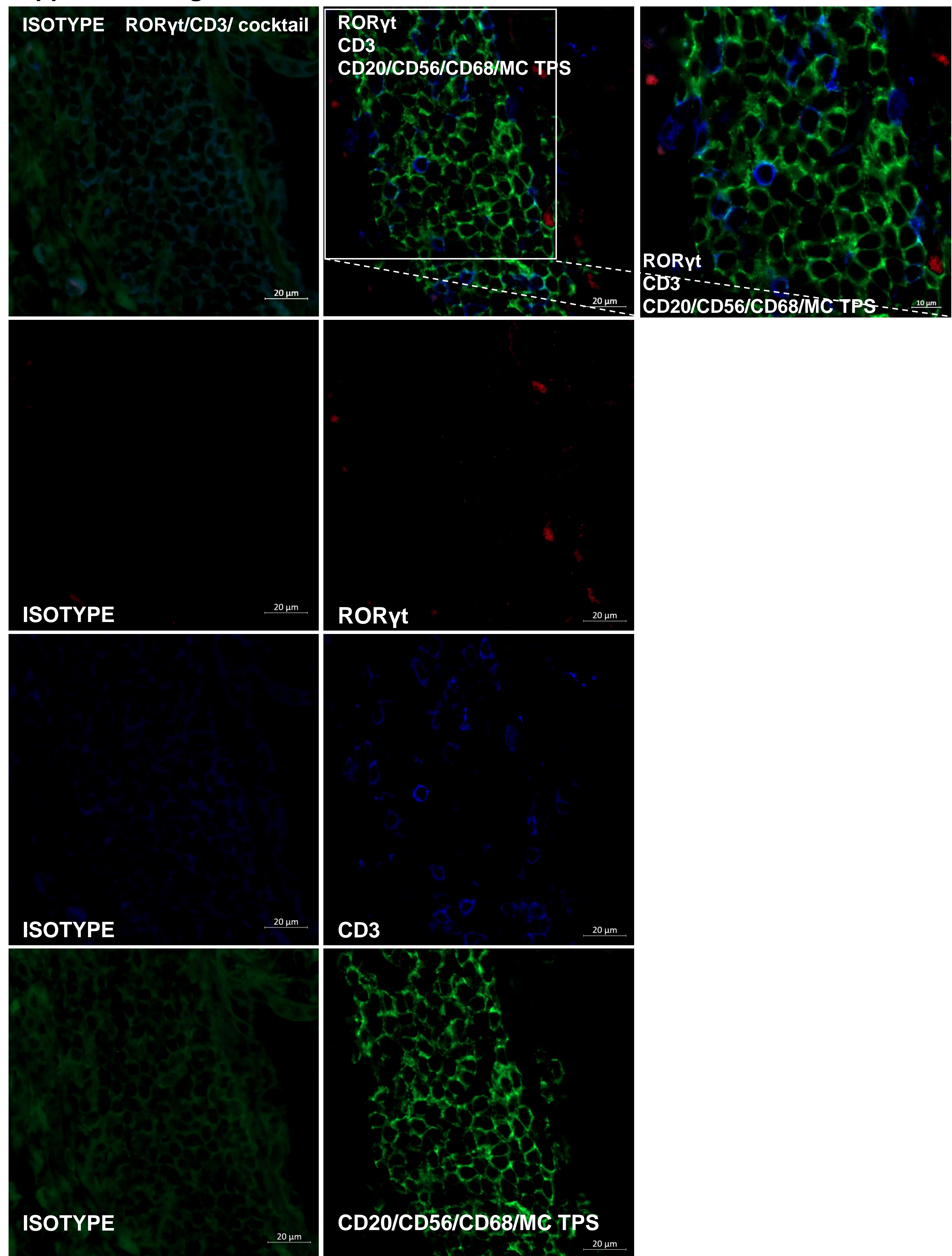
Supplementary figure 6
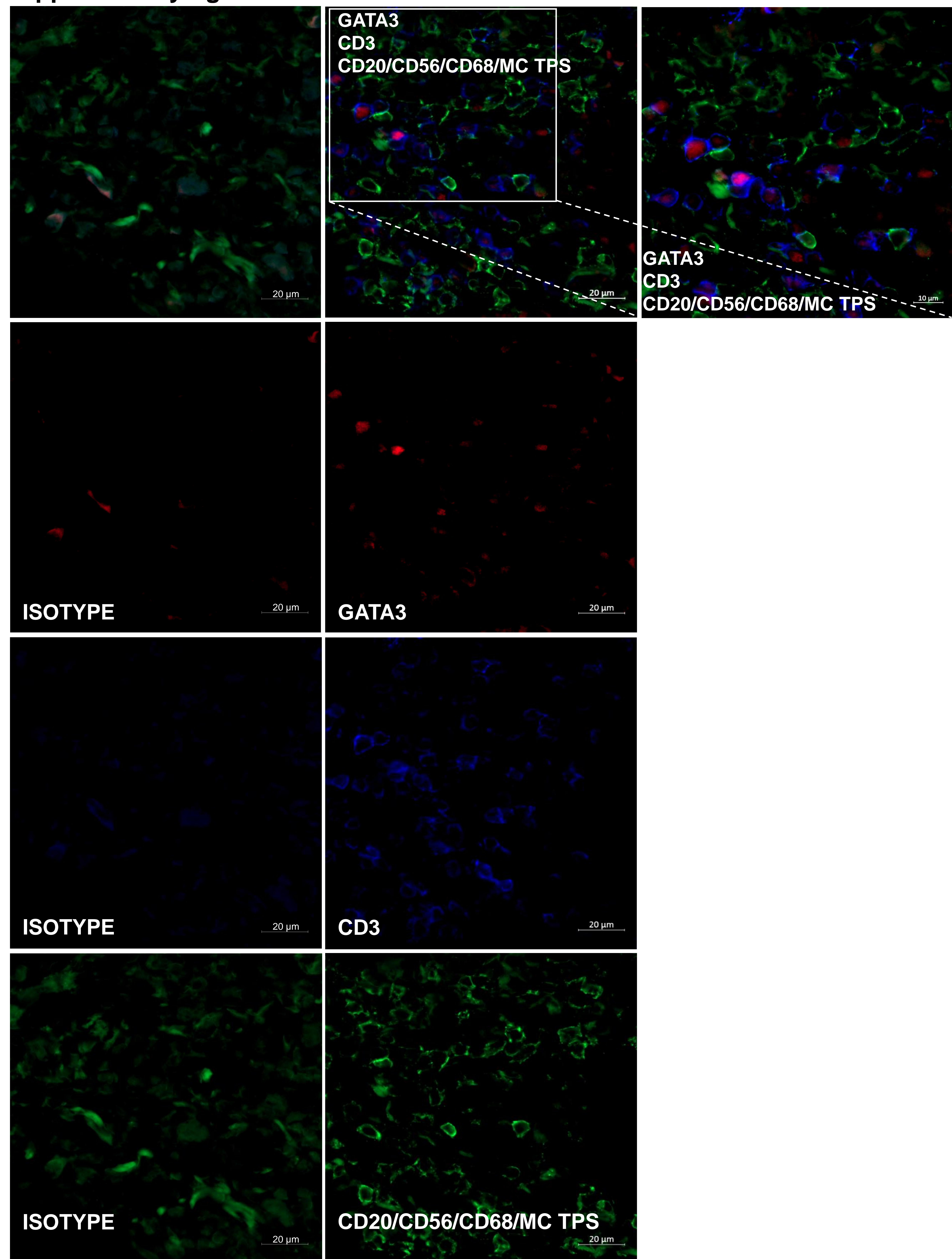
Supplementary figure 6: Isotype controls for immunofluorescent detection of pulmonary ILC and Th cells in COPD subject: left panels: Isotype-matched control antibodies for: GATA3, CD3 and the exclusion cocktail (CD20, CD56, CD68, mast cell tryptase), **middle and right panel**: staining for GATA3,CD3 and the exclusion cocktail. Rare staining artefacts and autofluoresence in isotype controls can clearly be distinguished from positive staining in the antibody stainings.
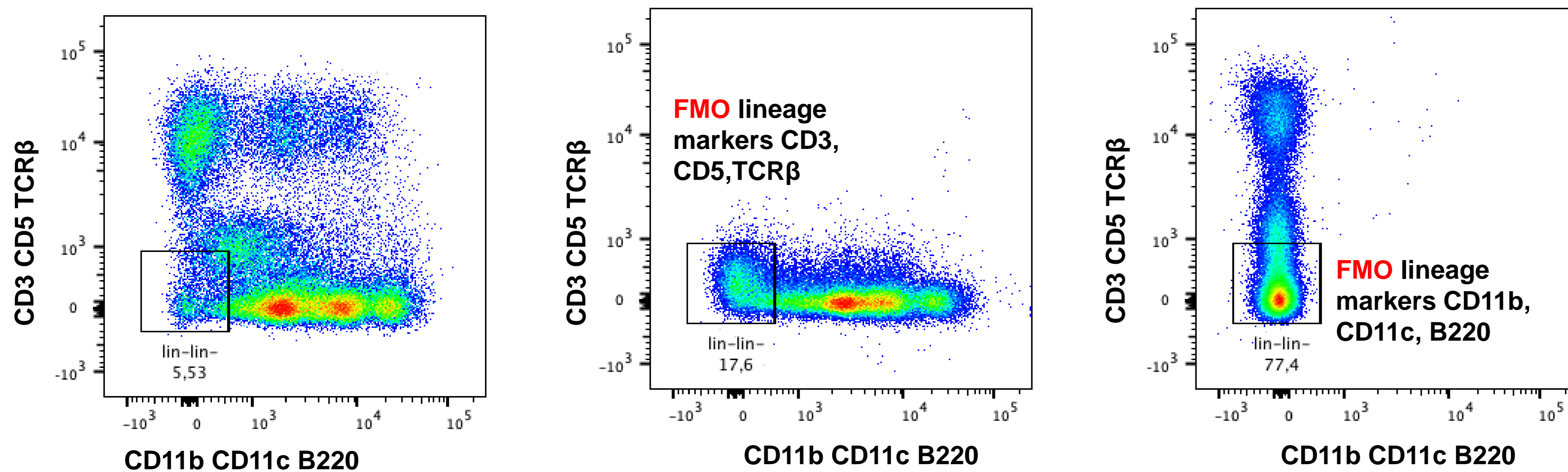
Supplemental figure 7

Supplementary figure 7: Isotype controls for immunofluorescent detection of pulmonary ILC and Th cells in COPD subject: **left panels:** Isotype-matched control antibodies for: RORγt, CD3 and the exclusion cocktail (CD20, CD56, CD68, mast cell tryptase), **middle and right panel**: staining for RORγt,CD3 and the exclusion cocktail. Rare staining artefacts and autofluoresence in isotype controls can clearly be distinguished from positive staining in the antibody stainings.
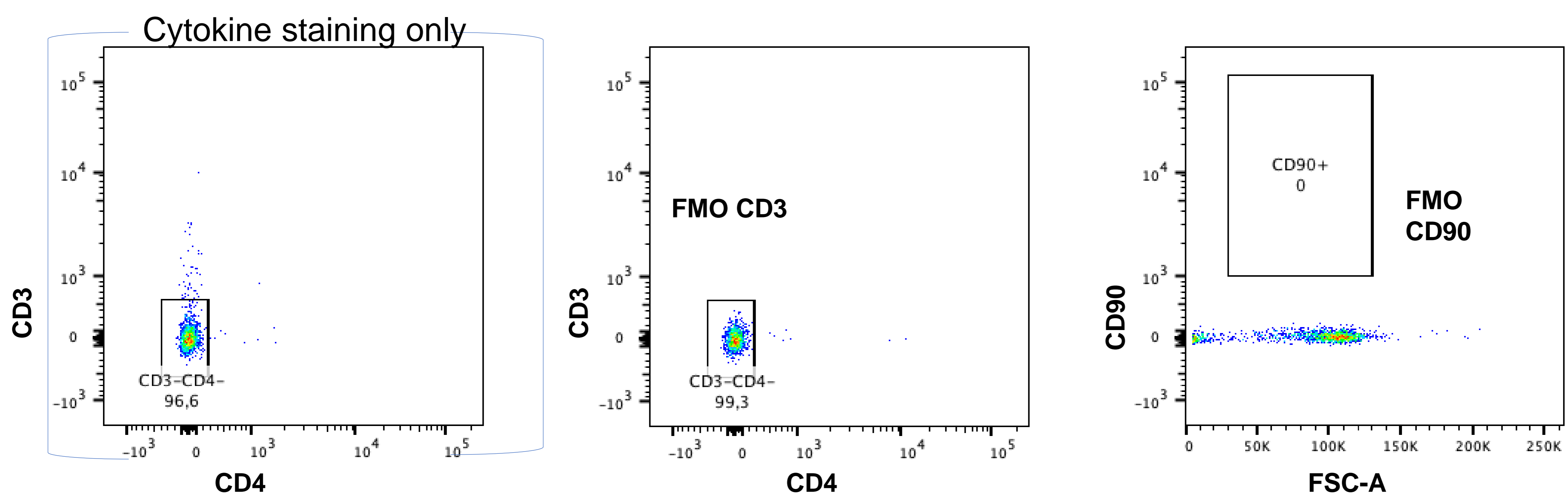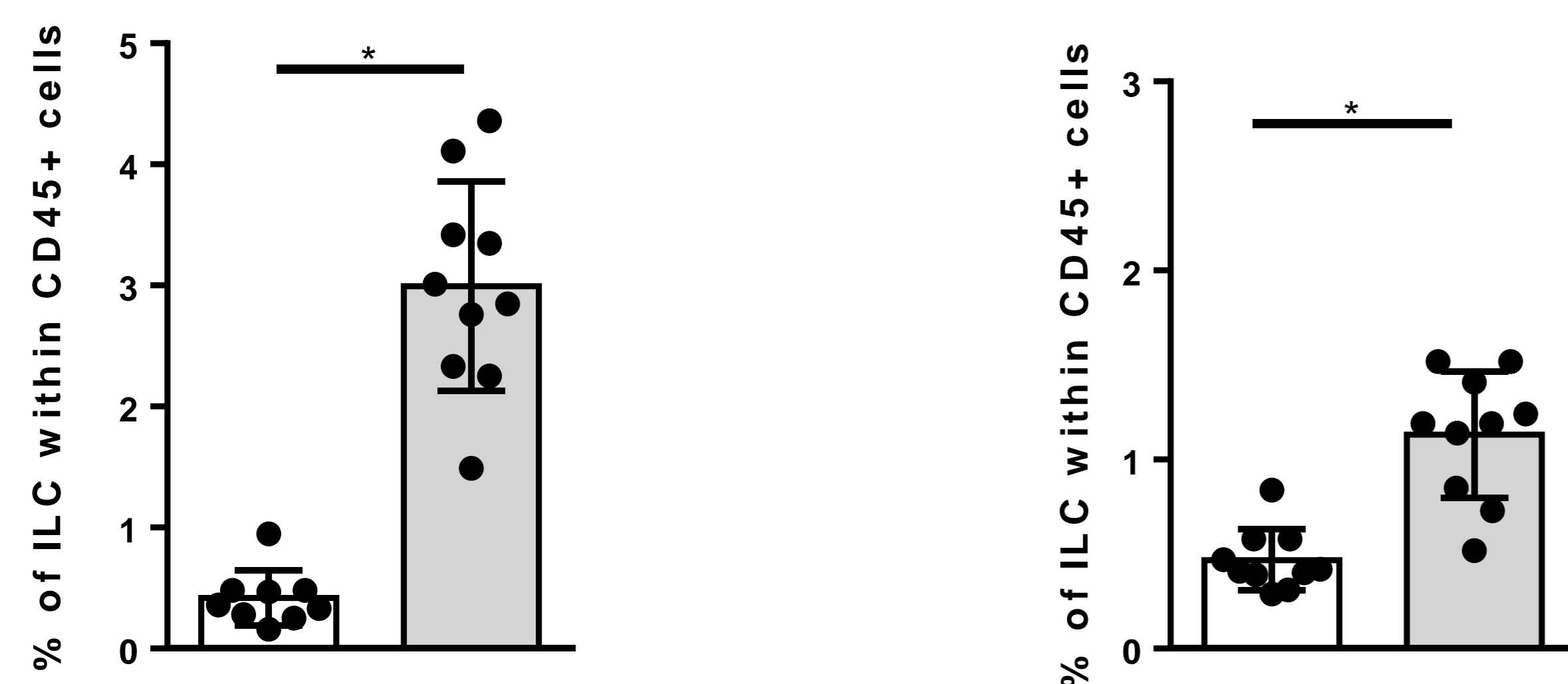
Supplementary figure 8

(a)

Within CD45+,live cells:

Within CD45+,live cells, lin-lin-:


(b)

4 weeks   24 weeks

Supplementary figure 8: FMOs and gating of Lineage markers, CD3 and CD90 for murine ILCs and % of ILC within CD45+ cells.

For flow cytometric analysis, ILC were surface stained and gated as live, CD45+ lymphocytes that are Lin-(CD3, CD5, TCRβ,CD11c, CD11b, B220) CD90+. For ILC labeling following 4h of stimulation (cytokine detection): ILC were gated as live, CD45+ lymphocytes that were Lin-(CD3, CD5, TCRβ,CD11c, CD11b, B220) CD3-CD4-CD90+.**(a)** % of CD90+ ILC within the CD45+ population in bronchoalveolar lavage fluid after exposure to air (white bars) or cigarette smoke (CS, grey bars) for 4- or 24-weeks. Results are expressed as mean ± SD. N: 8-10 mice per group. * P < 0.05.
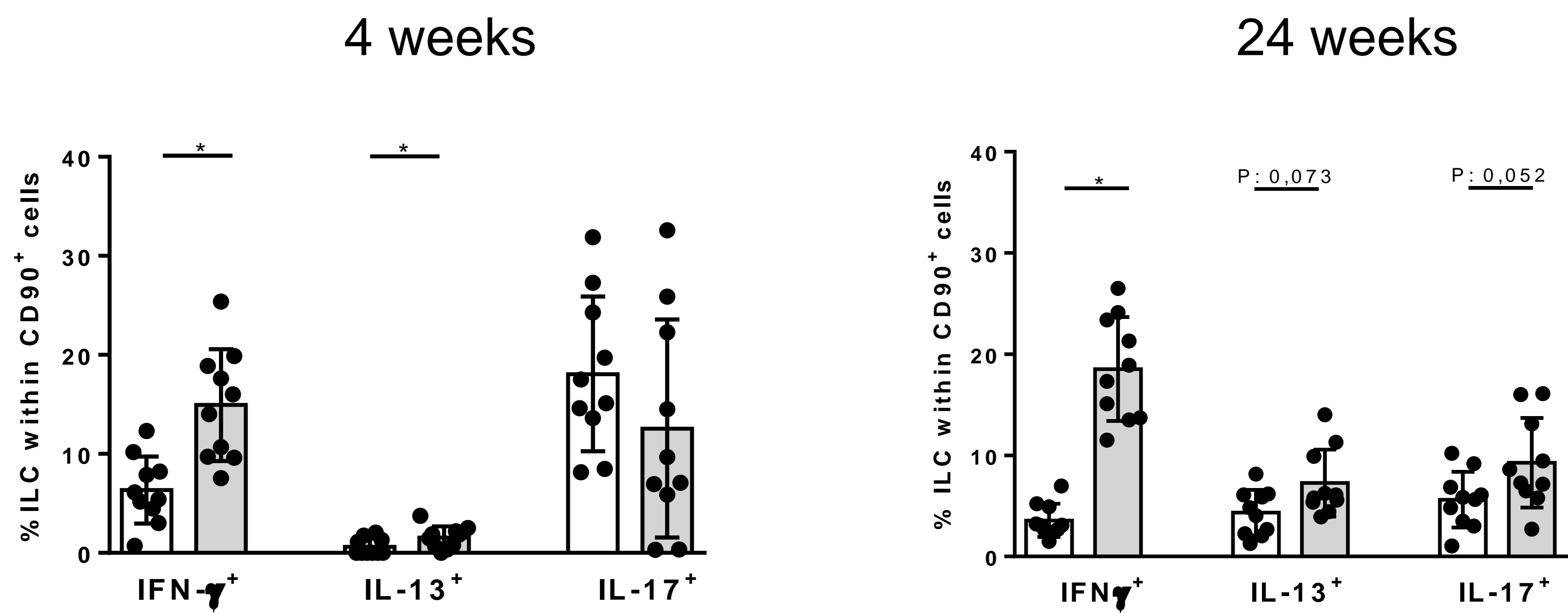
Supplementary figure 9

(a)

(b)
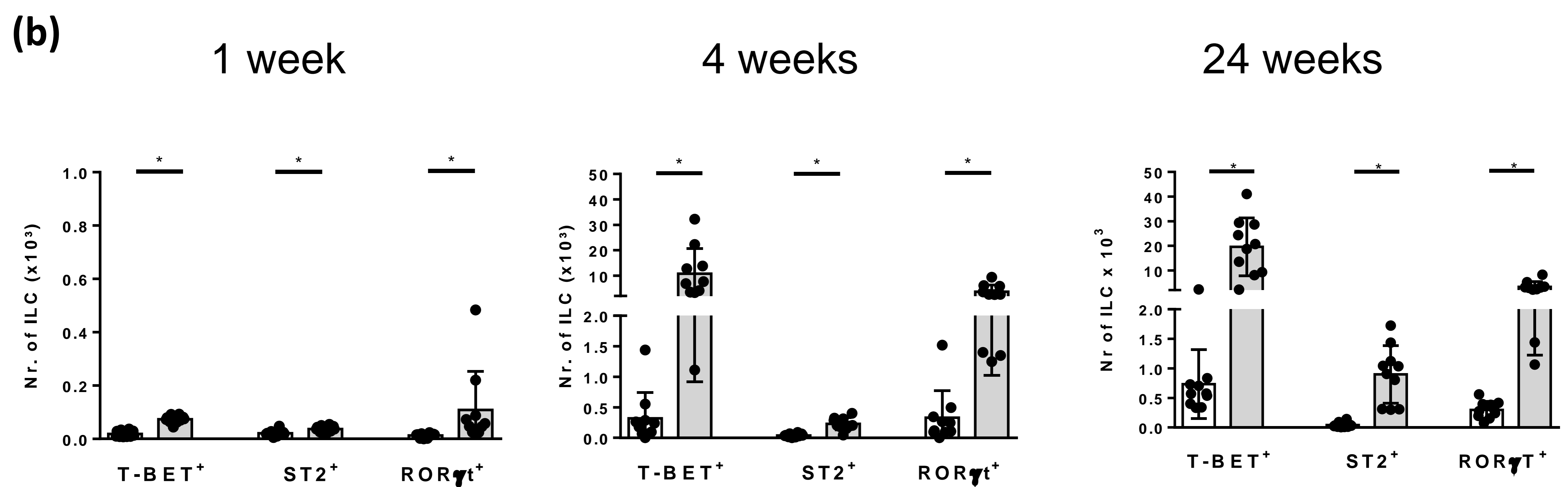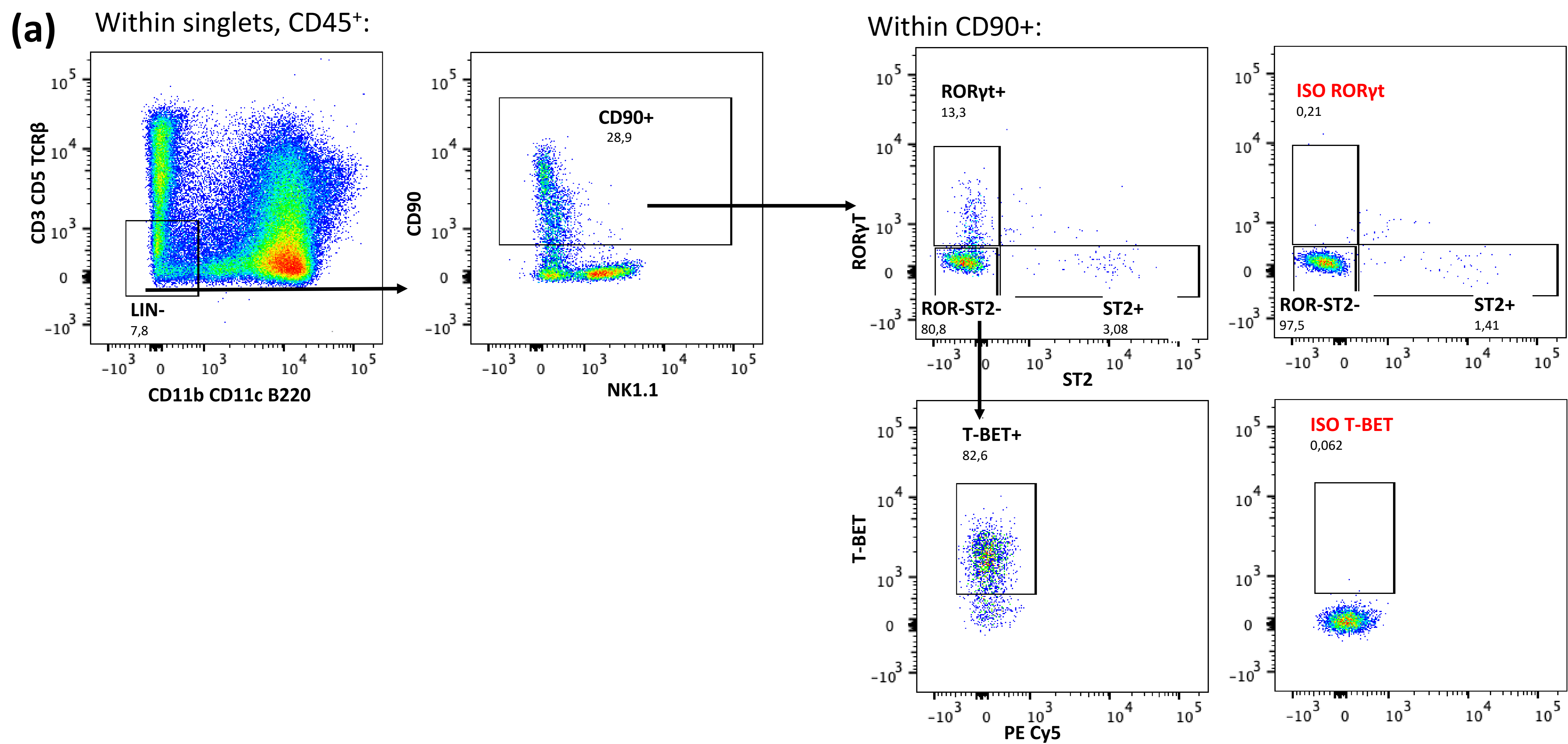
Supplementary figure 9: Distribution of ILC subsets in bronchoalveolar lavage fluid of mice after exposure to air or cigarette smoke.
For flow cytometric analysis, ILC were surface stained and gated as live, CD45$^+$ lymphocytes that are Lin$^-$(CD3, CD5, TCRβ, CD11c, CD11b, B220) CD90$^+$, followed by discrimination of the ILC subsets based on expression of T-BET, ST2 and RORγt (**a**). Numbers of ILC after exposure to air (white bars) or cigarette smoke (CS, grey bars) for 1, 4 and 24 weeks (**b**).

Supplementary figure 10

Supplementary figure 10: Relative distribution of IFNγ⁺ ILC, IL-13⁺ ILC and IL-17⁺ ILC within the CD90 ILC population in murine COPD model. Gating within the Singlets, CD45+, lin-, CD3-CD4-, CD90+ population. Numbers of ILC after exposure to air (white bars) or cigarette smoke (CS, grey bars) for 4 and 24 weeks.

Supplementary figure 11

(a)

(b)

(c)

(d)
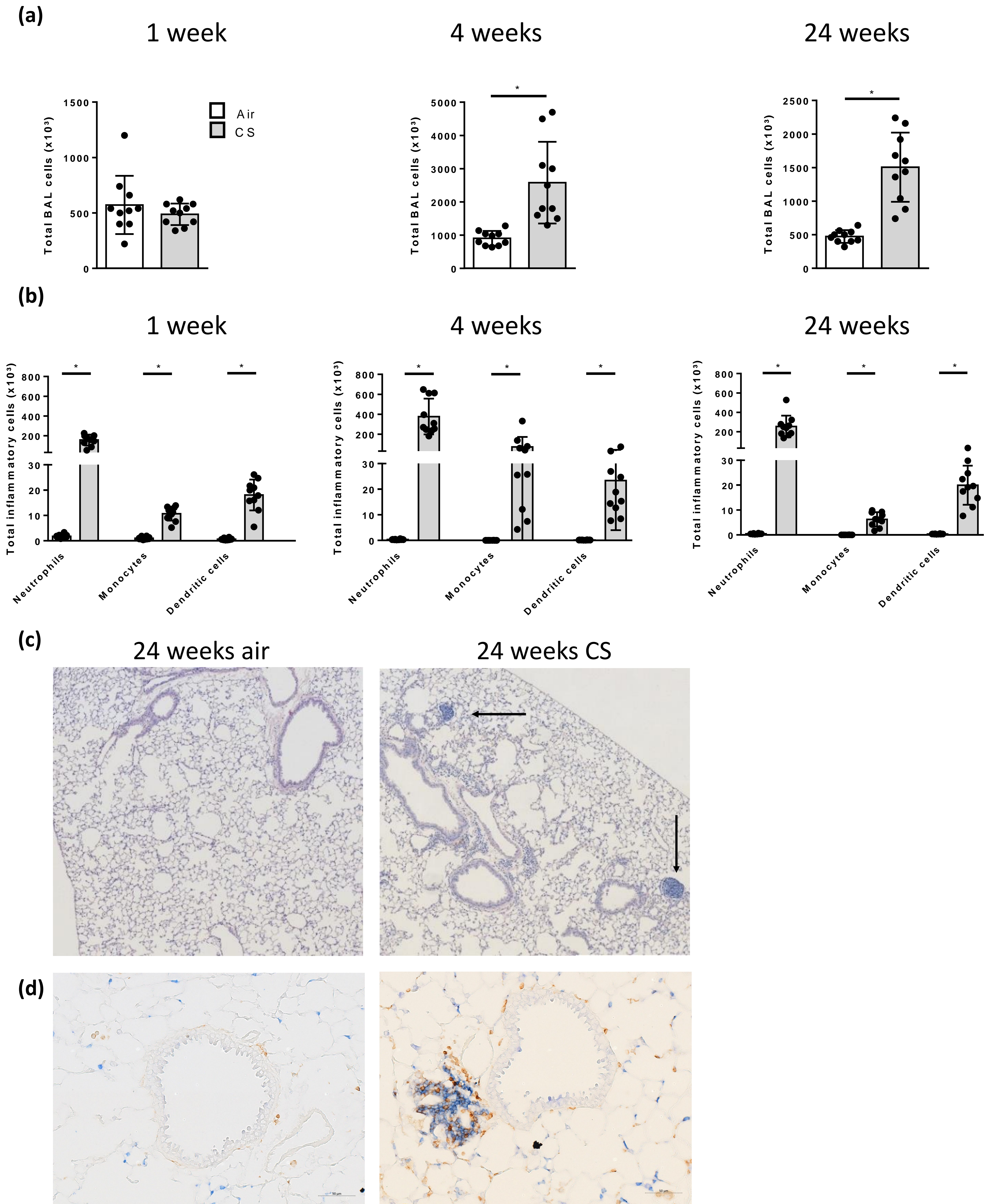
Supplementary figure 11: Cigarette smoke exposure induces innate inflammatory responses in mice. C57BL/6J mice were exposed to CS (grey bars) or air (white bars) for 5 days, 4 weeks or 24 weeks. Total numbers of cells in BAL **(a)**, $CD45^+CD11c^-CD11b^+Ly6C^+Ly6G^+$ neutrophils, $CD45^+CD11c^-CD11b^+Ly6C^+Ly6G^-$ monocytes and $CD45^+SiglecF^-CD11c^+MHCII^+CD11b^+$ dendritic cells in BAL **(b)**. Results are expressed as mean ± SD. N:10 mice per group. * P < 0.05. H&E staining **(c)** and CD3/B220 staining **(d)** of CS and air-exposed mice after 24 weeks, black arrows indicate lymphoid aggregates. Data from 5-day and 4-week experiments are representative of 2 experiments.