Appearance of Remodelled and Dendritic Cell-Rich Alveolar-Lymphoid Interfaces Provides a Structural Basis for Increased Alveolar Antigen Uptake in Chronic Obstructive Pulmonary Disease

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ONLINE DATA SUPPLEMENT
METHODS

Patients and Lung Tissue Sampling

Patients

Patients were classified as having COPD when FEV$_1$/FVC was less than 70%. The severity of COPD was based on FEV$_1$% of predicted according to GOLD criteria.[1] Lung tissue from 15 control subjects (never-smokers and smokers), 6 patients with GOLD stage I, 13 patients with GOLD stage II, and 2 patients with GOLD stage III COPD was obtained in relation to surgical resection (lobectomy) for suspected lung cancer.[2] Since it cannot be excluded that the cancer has influenced the immunological status of the lung we, in agreement with similar studies,[3-5] only included patients with a well-delineated bronchial tumour and restricted our analyses to only peripheral tissue as far away from the tumour as possible. Lung tissue from 10 patients with GOLD stage IV COPD was collected from patients requiring lung transplantation.[2] Patients with GOLD stage IV COPD had no history of lung cancer. None of the study subjects had $\alpha_1$-antitrypsin deficiency. For all patients, the surgical tissue sampling was performed outside any clinical exacerbation.

Peripheral tissue samples and lung compartments investigated for lymphoid aggregates (LAs)

Peripheral tissue samples from randomly selected lobes were subjected to standard fixation with 4% buffered paraformaldehyde. Care was taken to perform the fixation immediately after surgical excision and dissection. Next, the samples were dehydrated and prepared for histological analysis.[2] To allow a more direct comparison between the anatomical parts of the lung, tissue blocks containing bronchioles (non-cartilaginous, <2 mm in internal diameter), pulmonary vessels, and alveolar parenchyma were included in the study (importantly, the selection was performed on blinded sections, and at low power magnification with the sole purpose of assuring the presence of all anatomical regions). A
total of 102 tissue blocks were subjected to detailed analysis: 72 paraffin blocks (two blocks per subject) from controls and patients with GOLD stage I-III COPD, and 30 blocks (three blocks per subject) from patients with GOLD stage IV COPD. In total, among the study groups 978 bronchioles and pulmonary vessels were assessed for LAs. Of these 327 were bronchioles or bronchiole-associated vessels and 651 were solitary pulmonary vessels. There were no differences in numbers of analysed vessels or bronchioles between the study groups. Neither were there any differences in average vessel diameter or bronchiole perimeter between the study groups. For pulmonary vessels only pulmonary arteries, large arterioles, or veins with >140 µm in mean internal diameter were investigated for LAs. The mean vessel diameter was calculated by dividing the sum of the minimal and maximal endothelium-to-endothelium distance by two.

**Immunohistochemistry**

Prior to immunostaining, four-micron-thick paraffin embedded sections were heated at 60°C for 20 min, and subjected to simultaneous dewaxing and antigen retrieval according to table 2 (in main manuscript). All staining procedures were performed in an automated slide stainer (Autostainer Plus, DakoCytomation, Glostrup, Denmark). Antibody dilutions were prepared in Dako Antibody Diluent (S0809) and washing steps were performed using Dako Wash Buffer (K8007).

*Single staining immunohistochemistry*

Sections were immunostained with EnVision™ Peroxidase/DAB Detection System kit (Rabbit/Mouse K5007, Dako, Glostrup, Denmark) according to the manufacturer’s instructions. Briefly, endogenous peroxidase activity was blocked for 10 min by 0.3% hydrogen peroxide. The sections were incubated with primary antibodies directed against
CD20, cytokeratin, langerin, BDCA-2, Ki-67 or CD21 (table 2) for 1 h. For BDCA-2 staining, an additional incubation step with EnVision Flex+ Mouse (LINKER) (K8021) for 30 min was performed to amplify the signal of the antibody. All sections were then incubated with Polymer/HRP-linked secondary antibodies and the staining was visualised with 3,3’- diaminobenzidine (DAB) for 10 min. For BDCA-2 staining, sections were incubated with DAB substrate-chromogen solution for 40 min (10 min, four times). Next, sections were rinsed in distilled water and counterstained with Mayer’s haematoxylin, dehydrated through ethanol series, cleared in xylene and mounted with Pertex (HistoLab, Gothenburg, Sweden). The specificity of the immunostaining was evaluated by omitting primary antibodies. Immunostaining for CD20, langerin, BDCA-2 or CD21 was performed simultaneously on all tissue sections to avoid variability in staining intensity.

Double staining immunohistochemistry for the identification of lymphatic vessels

Lymphatic vessels were distinguished from smooth-muscle rich blood vessels using double staining immunohistochemistry for podoplanin (lymphatic vessel-specific marker) and α-smooth muscle actin (table 2). All paraffin sections were immunostained simultaneously. Endogenous peroxidase and alkaline phosphatase activity was blocked for 5 min using Dako Dual Endogenous Enzyme Block (S2003). Sections were then incubated for 1 h with antibodies directed against podoplanin, followed by incubation with Polymer/HRP-linked secondary antibodies (Dako EnVision™ Peroxidase/DAB Detection System kit, Rabbit/Mouse K5007). DAB chromogen was used to visualize the binding of the first antibody. Next, sections were incubated with alkaline phosphatase-conjugated anti-α-smooth muscle actin antibodies. The second staining was visualized using Liquid Permanent Red substrate kit (K0640, Dako). After rinsing in distilled water and counterstaining with Mayer’s haematoxylin, sections were mounted with Pertex.
Double staining immunohistochemistry for CD4 and langerin expression

Sections were immunostained with antibodies against CD4 using EnVision™ Peroxidase/DAB Detection System kit (Rabbit/Mouse K5007, Dako) as described above. Next, a blocking step with Denaturing Solution (DNS001H, L, Biocare Medical, Concord, CA, USA) for 5 min was performed to prevent additional binding to the first primary antibody. Sections were then incubated with Dako Protein Block Serum Free (X0909) for 10 min, followed by incubation with Dako Avidin/Biotin Blocking kit (X0590) for 10 min. Then sections were incubated with primary antibodies against langerin for 1 h, treated with biotinylated horse anti-mouse IgG secondary antibodies (1:200, BA-2000, Vector Laboratories, Inc., Burlingame, CA, USA), and incubated with Streptavidin-Alkaline Phosphatase (AP) (D0396, Dako). Immunoreactivity was detected with Liquid Permanent Red (K0640, Dako). Next, sections were counterstained with Mayer’s haematoxylin and mounted with Pertex.

Double staining immunohistochemistry for CD68 and CD11c expression

Double staining immunohistochemistry for CD68 and CD11c was performed using EnVision™ G2 Doublestain System (K5361, Dako) according to the manufacturer’s instructions. Endogenous peroxidase and alkaline phosphatase activity was blocked for 5 min. Sections were then incubated with antibodies against CD68, followed by incubation with Polymer/HRP-linked secondary antibodies and DAB substrate-chromogen solution. Next, a blocking step with Doublestain Block Reagent was performed to prevent additional binding of secondary antibodies to CD68 primary antibodies. Next, sections were incubated with antibodies against CD11c and then treated with polymer/AP-linked secondary antibodies. The
immunoreactivity was detected with Liquid Permanent Red. Finally, sections were counterstained with Mayer’s haematoxylin and mounted with Pertex.

**Double staining immunofluorescence**

Immunofluorescence was performed to investigate colocalization of langerin\(^+\) DCs and cytokeratin\(^+\) epithelial cells, and colocalization of langerin\(^+\) DCs and CD20\(^+\) B-cells. Sections were blocked for 10 min with Dako Protein Block Serum Free solution (X0909) to reduce non-specific binding. An additional blocking step with Dako Avidin/Biotin Blocking kit (X0590) for 10 min was performed, followed by incubation for 1 h with primary antibodies directed against langerin. Sections were then incubated for 1 h with biotinylated horse anti-mouse IgG secondary antibodies (1:200, BA-2000, Vector Laboratories), followed by 30 min incubation with Streptavidin-Alexa Fluor 555 (1:100, S32355, Life Technologies\({\text{TM/Molecular Probes}}^\circledR\), Eugene, OR, USA). Next, a blocking step with Denaturing Solution (DNS001H, L, Biocare Medical) for 5 min was performed to prevent additional binding of secondary antibodies to the first primary antibody. A second incubation for 1 h with primary antibodies directed against cytokeratin or CD20 was performed and antibody binding was detected by secondary Alexa Fluor 488 conjugated goat anti-mouse IgG antibodies (1:200, A11001, Life Technologies\({\text{TM/Molecular Probes}}^\circledR\)). Hoechst (H33342) was used as nuclear staining. Finally, sections were mounted in PBS/glycerine and examined using Nikon Eclipse 80i microscope (Nikon Instruments, Japan).

**Serial Section-Based Three-Dimensional Reconstruction of LAs**

Four-micron-thick serial sections (>400) were stained with Mayer’s haematoxylin-eosin. Digital images of whole sections were generated with ScanScope Slide Scanner (20x/40x objective, Aperio Technologies). LAs in 32 consecutive sections were manually traced. Images were captured using Aperio ImageScope software (Aperio Technologies) and saved to
an image stack of lossless .tiff file format. Registration and segmentation was done using Adobe® Photoshop CS 4™. The original image stack was registered by manually aligning consecutive slides. Segmentation was done by each LA of interest first being marked by histogram colour segmentation and then manually separated out of the original image and saved to a separate binary black and white file. The binary images for each segmented structure were then all reduced equally in image size maintaining aspect ratios and introduced as separate binary stacks into Amira® 5.4.2 Visage Imaging® (Visage Imaging Inc., San Diego, CA, USA). The 3D images were generated by overlaying each separate structure’s binary stack into the final composite image, maintaining X, Y, Z ratios between the stacks and adjusting for the section thickness in the Z voxel size. Similarly, series with 40 (four-micron-thick) serial sections stained with podoplanin antibodies was used to visualize the 3D relationship between lymphatic vessels, alveolar-LA interfaces and LAs.

**Quantifications**

High-resolution digital images of whole lung tissue were generated from all bright-field sections using ScanScope Slide Scanner (Aperio Technologies) and morphometric analyses were performed using Aperio ImageScope software (Aperio Technologies). All quantifications involving manual counting or manual cursor tracing was performed on blinded sections.

**Number of LAs**

The number and anatomical distribution of LAs were quantified on Masson’s trichrome-stained sections. On each section the perimeter of the whole lung tissue (including bronchioles, pulmonary vessels, parenchymal tissue, and airspaces) was manually traced and the corresponding cross-sectional area was expressed as square centimetre of the total
peripheral lung tissue. In all sections, the total number of LAs was counted and data were expressed as number of LAs per square centimetre of peripheral lung. Similarly, the number of LAs in the different lung compartments (i.e. small airway/bronchiolar- (BRALT), vascular- (VALT), and alveolar-only (ALT) associated aggregates) were counted and expressed as number of LAs per square centimetre of peripheral lung.

Cross-sectional area of LAs
The area of individual LAs was quantified on Masson’s trichrome-stained sections. On each section LAs were manually delineated and the number of pixels corresponding to the cross-sectional LA area was counted and expressed as square millimetre of the LA. The total cross-sectional area of all identified LAs in one section was calculated and data were expressed as the percentage of the LA area per total lung area.

Total CD20 immunoreactivity in whole sections
On each section, the perimeter of the whole lung tissue was delineated and a pixel threshold was selected to only include positive immunostained pixels (brown colour of DAB), representing CD20 immunoreactivity (Aperio Positive Pixel Count Algorithm v.9, Aperio Technologies). In the selected area, the total number of immunopositive pixels above the selected threshold level and the total number of pixels (i.e. immunopositive and negative) were used to calculate the percentage of the total lung area occupied by CD20-staining.

Alveolar-LA interfaces and surrounding anatomic structures
The number of LAs with a direct interface towards the alveolar lumen was quantified on podoplanin/α-smooth muscle actin-stained sections. The length of the alveolar-LA interface was measured by manual cursor tracing. The total length of alveolar-LA interfaces was
calculated for each section by multiplying the mean length of the alveolar interfaces (expressed in millimetres) by the number of LAs per square centimetre of peripheral lung. Data were expressed as the total length of alveolar interfaces per square centimetre of peripheral lung.

The length of podoplanin\(^+\) lymphatic vessels was measured by delineating only the part of the podoplanin\(^+\) vessel that surrounded the LA (i.e. podoplanin\(^+\) vessels within the LAs were excluded from the analysis). In addition to the alveolar lumen and lymphatic vessels, LAs were surrounded by airway wall compartments, the adventitia layer of pulmonary vessels and/or interstitial tissue, all of which were termed “tissue”. The length of each LA-associated structure, divided into alveolar interface, lymphatic vessel, or remaining firm tissue, was used to quantify the relative proportion of each anatomic structure along the LA perimeter.

*The epithelium at alveolar-LA interfaces*

In LAs with alveolar interfaces, the length of the interface epithelium was measured by manually tracing each type of epithelium (i.e. simple squamous, simple cuboidal or non-ciliated columnar epithelium). The proportion of squamous, cuboidal, or columnar epithelium was expressed as the percentage of the total alveolar interface length. The total length of the columnar interface epithelium was calculated for each section by multiplying the mean length of the columnar epithelium (expressed in millimetres) by the number of LAs per square centimetre of peripheral lung. The results were expressed as the total length of columnar epithelium per square centimetre of peripheral lung.

*Langerin\(^+\) DCs*

Langerin\(^+\) DCs within LAs (including the alveolar-LA interfaces) were manually counted and
expressed as number of langerin$^+$ DCs per square millimetre of LA. In LAs with alveolar
interfaces, the number of langerin$^+$ DCs along squamous, cuboidal or columnar interface
epithelium was counted and the data were expressed as the number of DCs per millimetre
length of the interface epithelium. Similarly, the numbers of BDCA-2$^+$ plasmacytoid and
CD68$^-$CD11c$^+$ myeloid DCs were quantified along the alveolar-LA interfaces. The total
number of interface langerin$^+$ DCs (including squamous, cuboidal and columnar interface
epithelium) was calculated on each section by multiplying the mean number of langerin$^+$ DCs
per millimetre alveolar interface by the number of LAs per square centimetre of peripheral
lung. Data were expressed as the total number of interface langerin$^+$ DCs per square
centimetre of peripheral lung.

In LAs with alveolar interfaces, the number of langerin$^+$ DCs was quantified in three separate
regions within the LAs: the superficial region, defined as the region within a distance of 20
µm beneath the alveolar-LA interface; the central part of the LA; the opposing region, defined
as the region within a distance of 20 µm beneath the boarder of surrounding firm tissue. The
area of the superficial region was calculated by multiplying the length of the alveolar interface
by 20 µm and data were expressed as number of DCs per square millimetre of the superficial
region. The number of DCs in the central part of the LA was counted within a grid (20 µm x
98 µm) and the data were expressed as average number of DCs per square millimetre grid
area. The area of the opposing region was calculated by multiplying the length of the tissue
boarder by 20 µm and data were expressed as number of DCs per square millimetre of the
opposing region.

RESULTS

Patients
A complete medical history was obtained from 9 out of 10 patients in the GOLD stage IV-group. There was no difference in age between control subjects and patients with COPD (p>0.05). Moreover, there was no difference in pack-years between smokers without COPD and patients with COPD (p>0.05).

**General Expansion and Characteristics of LAs in Advanced Stage COPD**

Peripheral lung LAs were observed in all study groups (figure S1A and table S1). Advanced COPD was associated with both increased number of LAs per tissue area and percentage of the total lung tissue occupied by LAs compared with never-smoking control subjects (figure S1A,B). There was no difference in the mean cross-sectional area of LAs between the control and COPD groups (table S1). Since the vast majority of the CD20⁺ B-cells in COPD lungs are localized to LAs (exemplified in figure S1D), total tissue CD20⁺ cells was used as complementary marker of lung LAs. Also this parameter increased in advanced COPD (figure S1C).

Cells positive for the proliferation antigen Ki-67 and CD21, a marker of follicular DCs, (figure S2) were used as indices of maturation and activation status of the lung LAs. The percentage of LAs containing CD21⁺ follicular DCs was significantly increased in patients with GOLD stage IV COPD (median, 100%; range, 53-100%) compared with never-smoking controls (median, 20%; range, 0-50%; p=0.006). Although the expression of Ki-67 was not quantified, it was evident that clusters of Ki-67⁺ cells were commonly observed among the LAs.

Importantly, neither the numbers of BDCA-2⁺ plasmacytoid, nor CD68⁻CD11c⁺ myeloid DCs were found to have similar spatial association to alveolar-lymphoid interfaces as the langerin⁺ DCs (figure S3).
FIGURE LEGENDS

Figure S1. Expansion of peripheral lymphoid aggregates (LAs) in patients with COPD. 

(A) The total number of LAs and (B) the total cross-sectional area of LAs were quantified on Masson’s trichrome-stained sections from peripheral lung samples obtained from never-smokers, smokers without COPD and patients with GOLD stage I-IV COPD. (C) Quantification of the total tissue immunoreactivity for CD20<sup>+</sup> B-cells. (A-C) Statistical analyses were performed using Kruskal-Wallis nonparametric test followed by Dunn’s multiple comparison post-test. Horizontal lines indicate medians for each group. *: p<0.05; **: p<0.01. (D) The vast majority of CD20<sup>+</sup> B-cells (brown colour) were located within scattered LAs that were distributed throughout the distal lung, including (E) bronchioles (Br), (F) pulmonary vessels (PV, black pigment deposition is visible), and (G) the alveolar parenchyma. Cell nuclei were counterstained with Mayer’s haematoxylin (blue stain). Scale bars: (D) 1000 µm; (E-G) 100 µm.
Figure S2. Characterisation of lymphoid aggregates (LAs) in patients with GOLD stage IV COPD. (A) Ki-67\(^+\) proliferating cells (shown in brown colour) were frequently present in the LA centres (i.e. germinal centres). (B) CD21\(^+\) follicular dendritic cells (brown colour) formed a network within the LAs. (C) A higher magnification of the CD21\(^+\) follicular dendritic cell network. Cell nuclei were counterstained with Mayer’s haematoxylin (blue stain). Scale bars: (A-B) 150 µm; (C) 50 µm.
Figure S3. Comparisons between dendritic cell (DC) subsets along alveolar-lymphoid aggregate (LA) interfaces. (A) Quantification of the numbers of BDCA-2+ plasmacytoid DCs, CD68−CD11c+ myeloid DCs, and langerin+ DCs along the alveolar-LA interfaces. Values are given as mean ± SEM. (B) Photomicrograph exemplifying the distribution of BDCA-2+ plasmacytoid DCs (brown colour) in LAs. Black pigment deposition is visible. (C) Photomicrograph exemplifying CD68+ monocytes/macrophages (brown colour) and CD68− CD11c+ myeloid DCs (red colour) in LAs. Cell nuclei were counterstained with Mayer’s haematoxylin (blue stain). Scale bars: (B-C) 50 µm.
### TABLE S1. CHARACTERISTICS OF LYMPHOID AGGREGATES IN DIFFERENT ANATOMICAL COMPARTMENTS OF PERIPHERAL LUNG

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Never-smokers</th>
<th>Smokers w/o COPD</th>
<th>GOLD I COPD</th>
<th>GOLD II-III COPD</th>
<th>GOLD IV COPD</th>
<th>Overall p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects with LAs (yes/no)</td>
<td>5/3</td>
<td>5/2</td>
<td>4/2</td>
<td>13/2</td>
<td>10/0</td>
<td>0.249</td>
</tr>
<tr>
<td>Number of LAs per cm² of total lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRALT</td>
<td>0.51 (0.2-2.27)</td>
<td>0.85 (0.637)</td>
<td>0.51 (0.486)</td>
<td>0.55 (0.617)</td>
<td>2.67 (0.40-3.98)</td>
<td>0.071</td>
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<tr>
<td>VALT</td>
<td>0 (0-0.42)</td>
<td>0 (0-2.55)</td>
<td>0.51 (0.680)</td>
<td>0 (0.124)</td>
<td>1.35 (0.43-3.27)</td>
<td>0.001</td>
</tr>
<tr>
<td>ALT</td>
<td>0 (0.181)</td>
<td>0 (0.527)</td>
<td>1.92 (0.86)</td>
<td>1.16 (0.852)</td>
<td>3.21 (0.76-5.05)</td>
<td>0.052</td>
</tr>
<tr>
<td>Total LA area, % of total lung area</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRALT</td>
<td>0.01 (0-0.09)</td>
<td>0.02 (0-0.21)</td>
<td>0.01 (0-0.10)</td>
<td>0.01 (0-0.24)</td>
<td>0.10 (0.01-0.18)</td>
<td>0.027</td>
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<tr>
<td>VALT</td>
<td>0 (0-0.01)</td>
<td>0 (0-0.08)</td>
<td>0.01 (0-0.21)</td>
<td>0 (0-0.05)</td>
<td>0.06 (0.01-0.15)</td>
<td>0.001</td>
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<tr>
<td>ALT</td>
<td>0 (0-0.02)</td>
<td>0 (0-0.22)</td>
<td>0.03 (0-0.61)</td>
<td>0.03 (0-0.59)</td>
<td>0.05 (0.02-0.16)</td>
<td>0.021</td>
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<tr>
<td>Cross-sectional area of LAs, mm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All LAs</td>
<td>0.02 (0.01-0.04)</td>
<td>0.03 (0.01-0.05)</td>
<td>0.02 (0.01-0.16)</td>
<td>0.02 (0.01-0.04)</td>
<td>0.03 (0.02-0.17)</td>
<td>0.246</td>
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<tr>
<td>BRALT</td>
<td>0.02 (0.01-0.08)</td>
<td>0.03 (0.02-0.05)</td>
<td>0.01 (0.01-0.02)</td>
<td>0.03 (0.01-0.04)</td>
<td>0.03 (0.01-0.05)</td>
<td>0.318</td>
</tr>
<tr>
<td>VALT</td>
<td>0.01 (0.01-0.03)</td>
<td>0.04 (0.02-0.06)</td>
<td>0.02 (0.01-0.10)</td>
<td>0.04 (0.01-0.05)</td>
<td>0.04 (0.02-0.06)</td>
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<tr>
<td>ALT</td>
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<td>0.02 (0.01-0.04)</td>
<td>0.02 (0.01-0.34)</td>
<td>0.02 (0.02-0.05)</td>
<td>0.03 (0.01-0.05)</td>
<td>0.608</td>
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</tbody>
</table>

Values are median (range), unless specified by n.
* Kruskal-Wallis test for differences between groups. Dunn’s multiple comparisons post-test: † p<0.05 compared with never-smokers; †† p<0.01 compared with never-smokers; ‡ p<0.05 compared with GOLD stages II-III COPD; ‡‡ p<0.01 compared with GOLD stages II-III COPD.
ALT, alveolar-only lymphoid tissue; BRALT, small airway/bronchiolar-associated lymphoid tissue; COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease; LA, lymphoid aggregate; VALT, vascular-associated lymphoid tissue.

### TABLE S2. CORRELATIONS TO FEV₁/(F)VC

<table>
<thead>
<tr>
<th>Variable</th>
<th>All COPD Subjects</th>
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<tbody>
<tr>
<td></td>
<td>r-value</td>
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<tr>
<td>Total length of alveolar interfaces (mm) per cm² lung</td>
<td>-0.46</td>
</tr>
<tr>
<td>Total length of columnar epithelium (mm) per cm² lung</td>
<td>-0.70</td>
</tr>
<tr>
<td>Percent CD20+ area of total lung area</td>
<td>-0.46</td>
</tr>
<tr>
<td>Total number of interface langerin+ dendritic cells per cm² lung</td>
<td>-0.49</td>
</tr>
</tbody>
</table>

* Spearman’s rank correlation was used.
COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in one second; (F)VC, (forced) vital capacity.
REFERENCES


