Distribution of TRPC5 in the human lung: A study in body donors

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Abstract. Transient receptor potential channel canonical 5 (TRPC5) is a non-selective ion channel; ion influx through TRPC5 causes activation of downstream signaling pathways. In addition, TRPC5 has been identified as having a potential role in pathological processes, particularly in diseases caused by cellular cation homeostasis dysregulation, such as bronchial asthma or pulmonary hypertension. However, the expression and distribution of TRPC5 in the human lung remain unclear. To date, TRPC5 has only been detected in a few cell types in the human lung, such as airway, pulmonary venous and arterial smooth muscle cells. The present study therefore aimed to investigate the protein expression of TRPC5 in the human lung and to evaluate its histological distribution. Human lung samples were obtained from six preserved body donors. After processing, both hematoxylin & eosin staining, as well as immunohistochemistry were performed. Microscopic analysis revealed medium to strong immunostaining signals in all lung structures examined, including the pleura, pulmonary arteries and veins, bronchioles, alveolar septa, type 1 and 2 pneumocytes, as well as alveolar macrophages. Current research suggests that TRPC5 may be involved in various pathological processes in the human lung and some pharmacological compounds have already been identified that affect the function of TRPC5. Therefore, TRPC5 may present a novel drug target for therapeutic intervention in various lung diseases. The results of the present study indicate that the TRPC5 protein is expressed in all examined histological structures of the human lung. These findings suggest that TRPC5 may be more important for physiological cell function and pathophysiological cell dysfunction in the lung than is currently known. Further research is needed to explore the role and therapeutic target potential of TRPC5 in the human lung.

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Introduction

The transient receptor potential channel canonical 5 (TRPC5) is localized in the cell membrane and capable of conducting mono- and divalent ions such as Na^+, Ca^{2+}, or Mg^{2+} along their electrochemical gradient into the cell (1). By doing so, TRPC5 can depolarize excitable cells or activate various downstream signaling pathways (1,2). The channel can be activated by signaling pathways or other stimuli such as mechanical stress or ligands (1,2). Therefore, TRP channels, to which TRPC5 belongs, are often referred to as multimodal sensory proteins (1). Next to the canonical (TRPC) subfamily, the polycystin (TRPP), mucolipin (TRPML), vanilloid (TRPV), melastatin (TRPM), and ankyrin (TRPA) have been described. TRP channels share structural similarities and are typically tetrameters. Each monomer consists of six transmembrane domains with an amino- and carboxyl-terminal end that extends into the cytoplasm. Among the different groups and members, these endings exhibit major structural differences (1,2).

The most important mechanism of stimulation of TRPC5 is the activation through G proteins and phospholipases C (3,4). While the function of TRPC5 as a store-operated channel is still being discussed, TRPC5 seems to be a major regulator of cellular Ca^{2+}-homeostasis (3,5). In smooth muscle cells, TRPC5's role as a regulator of cellular Ca^{2+}-homeostasis is critical for cell functionality (6). TRPC5 has been reported in different localizations that include the central nervous system, the kidney, and the cardiovascular system (3,5). In the lung, TRPC5 has been detected in smooth muscle cells, of airways and of venous and arterial blood vessels, as well as in neuroepithelial bodies of the intrapulmonary airway (7–11). In their study, Peng *et al* (7) isolated rat pulmonary venous smooth muscle cells and detected TRPC5 messenger ribonucleic acid (mRNA) by performing real-time polymerase chain reaction (RT-PCR). Similarly, Lu *et al* (8) detected TRPC5 mRNA in rat pulmonary arterial smooth muscle cells. White *et al* (9) detected TRPC5 in human airway smooth muscle cells through western blot analysis and the corresponding mRNA through RT-PCR. In airway smooth muscle cells of guinea pigs TRPC5 mRNA has also been detected using RT-PCR by Ong *et al* (10). Finally, Lembrechts *et al* (11) detected the TRPC5-protein using immunohistochemical staining in neuroepithelial bodies of mice in the intrapulmonary airway epithelium. These are clusters of pulmonary neuroendocrine

cells (12). Additionally, there is also evidence that TRPC5 is present in macrophages (13). Tao *et al* (14) provided evidence suggesting that TRPC5 in macrophages may inhibit the polarization into a proinflammatory M1 phenotype, by showing that macrophages of the M1 phenotype were increased in the aortic wall of TRPC5-knockout mice. Another study, conducted by Pereira *et al* (15) also revealed that TRPC4/TRPC5 complexes in macrophages may mediate a protective role in sepsis. Although TRPC5 appears to be important for the physiological function of macrophages, to our knowledge, it has not been described in lung-specific alveolar macrophages.

A recent study, conducted by Yang *et al* (16) provided evidence that TRPC5 promotes proliferation and invasion of cancer cells through the upregulation of the hypoxia-inducible factor-1α (HIF-1α) signaling pathway in papillary thyroid carcinoma. Similar findings in colon carcinoma cells were presented by Chen *et al* (17). Furthermore, overexpression of both TRPC5 and dysregulation of cellular Ca^{2+}-homeostasis are associated with higher levels of p-glycoproteins (p-GP) in the plasma membrane of cancer cells. P-GP are associated with the outward transfer of chemotherapeutic drugs like paclitaxel or adriamycin. Thus, TRPC5 may play an important role in the development of chemoresistance in cancer cells (18). To our knowledge, the role of TRPC5 in lung cancer has not been subjected to extensive research yet.

In summary, TRPC5 has already been detected in many cell types as a critical player in physiological processes (3). While some studies have already detected TRPC5 in the lung, only a few cell types have been examined. Most studies used single cell cultures, animal tissue, or detected the levels of TRPC5 mRNA and not the protein directly. Furthermore, many studies focused on the function and regulation of TRPC5. While these aspects are important, knowledge of the localization of TRPC5 in the human lung is critical to acquire a better understanding of the channel. Therefore, the aim of the present study was to detect the TRPC5-protein in the human lung using immunohistochemistry and to determine its histological distribution profile.

Materials and methods

Specimens. All samples used in this study originated from voluntary body donors (n_1=6) from the body donation program of Saarland University's Institute for Anatomy and Cell Biology. All samples were collected in April 2023. All sexes, ages, body mass indices (BMI), and causes of death were susceptible to inclusion. No specific exclusion criteria were applied. While four of the body donors were male, two were female. All donors died of natural causes. One donor died of pneumonia. Due to limited data, we could not evaluate the bacteria that caused the infection or whether the pneumonia was community- or hospital-acquired. The body donor's characteristics are displayed in Table I. Following their decease, the body donors were preserved using nitrite pickling salt and ethanol (NEP) according to the protocol used in a previous study conducted by Janczyk *et al* (19). There is evidence that NEP preservation causes less protein denaturation and retains antigenicity better than formaldehyde-based preservation (20,21). After NEP fixation, a median thoracotomy was performed to access the donor's lung. From each donor, we collected 4 samples from different regions of the lung, including the left and the right apex, the basal region of the right lung, and the central region near the hilus of the right lung. Out of these 24 samples, 10 (n_2=10) were selected for immunostaining; For tissue comparisons between donors, samples taken from the left apex of each doner's lung were selected as they provided the best overall tissue quality of each extraction site (Fig. 1A-F). Similarly, all samples taken from donor 5 were selected to evaluate differences between extraction sites. To further evaluate the difference between peripheral and central extraction sites, an additional sample from the hilus-near extraction site of donor 4 was selected.

For positive control, we gained tissue samples of the heart, as TRPC5 has previously been detected in that tissue (22). Afterwards, the samples were processed using 4% formaldehyde and embedded in paraffin as described before (23). A tissue thickness of 6 µm was obtained using a microtome. Slices were then placed onto microscope slides.

Immunohistochemistry. Hematoxylin & eosin (H&E) staining was performed as previously described by Diebolt *et al* (23). Fig. 1 shows representative images of H&E-stained samples from each body donor. For immunostaining, heat-induced epitope retrieval (HIER) was performed to ensure complexing of the antibody with the antigen (24). The samples were placed in 1% citrate-buffer solution and incubated at 95˚C for up to 60 min. After HIER, the samples were washed in phosphate-buffered saline (PBS) and normal goat serum (NGS), used as blocking solution, was added (cat. no. 01-6201; Invitrogen AG; Thermo Fisher Scientific, Inc.). To ensure proper binding of the knockout-validated primary anti-TRPC5 antibody (cat. no. ACC-020; Alomone Labs) solution (1:50 in NGS), samples were incubated for 12 h at room temperature. The negative control group was incubated with rabbit serum instead (Institute for Medical Biochemistry and Molecular Biology, Saarland University). The samples were then washed again with PBS and incubated in a 3% H_2O_2 solution for 10 min to deactivate the endogenous peroxidases. Goat anti-rabbit antibody, in conjunction with horseradish peroxidase (1:500 in NGS), was added (cat. no. A10547 Invitrogen AG; Thermo Fisher Scientific, Inc.). The samples were incubated for 45 min at room temperature and washed with PBS. In order to visualize the antibody binding sites, we incubated the samples with diaminobenzidine (DAB) for 10 min (cat. no. SK-4103; Vector Laboratories, Inc.). The samples were counterstained with hematoxylin before cover slipping.

Analysis. The anti-TRPC5-stained samples (n_2=10) were analyzed under a light microscope equipped with a digital camera (MikroCam SP 5.1; Bresser, GmbH). The staining signals were then classified into three groups, based on their intensity, and ranging from no staining signal to medium and strong staining signals. Samples with no staining signal will only display the blue-colored counterstain. Samples with a light to dark orange color are considered to have a medium staining signal, the blue counterstain may still be visible in these samples. Strongly stained samples present a light to dark

Table I. Body donors' characteristics.

Body donor	Sex	Age, years	Date of death, month/year	Time between death and preservation, days	Cause of death	Further clinical conditions
1	M	83	03/2023	1	Metastatic prostate carcinoma	Arterial hypertension, pulmonary embolism (S/P)
2	M	92	03/2023	2	Pneumonia	Renal failure, aortic valve replacement (S/P)
3	F	80	03/2023	4	Upper gastrointestinal bleeding	Cardiac arrhythmia, coronary artery disease
4	M	81	03/2023	4	Multi-organ failure	Metastatic urothelial carcinoma
5	F	79	03/2023	4	Heart failure	Absolute tachyarrhythmia
6	M	72	03/2023	3	Multi-organ failure	Pulmonary insufficiency, diabetes mellitus type II

M, male; F, female; S/P, status post.

brown color; In these samples, the blue counterstain is almost no more visible. Subsequently, H&E- and immunohistochemically-stained samples were digitalized by using the Nano Zoomer S210 (Hamamatsu, Japan) to obtain representative high-resolution images (Figs. 1 and 2).

Results

To confirm that the samples' histological structures were intact, hematoxylin-eosin staining (H&E) was performed. The overall histologic structure of all samples was intact. Alveoli, blood vessels, and bronchioles were recognizable. Proteolysis was limited and artifacts such as tissue detachment were minimal. Donors 1, 2, 4, and 5 presented an emphysematous enlargement of the alveoli (Fig. 1A, B, D, and E). In addition, eosinophilic exudate was detectable in the alveoli of donor 1 (Fig. 1A). Samples taken from donor 2 show slight signs of infiltrating immune cells in the subpleural connective tissue. Bacterial infiltration is not visible (Fig. 1B). Donors 3, 5, and 6 showed signs of fibrotic remodeling of the interalveolar septa and subpleural tissue (Fig. 1C, E, and F). Furthermore, the incorporation of anthracotic pigments into the connective tissue of the lung was identifiable in all donors. There were no signs of malignant cell infiltration in any sample. Sample quality was ultimately deemed acceptable, as the general histologic lung structure of donors 1-6 was intact and artifacts due to tissue processing and embedding were minimal.

In the overview of anti-TRPC5-stained samples (n_2=10), two presented an overall strong and eight a medium staining signal, while none presented a negative staining signal.

Further analysis, revealed that all major histological structures of the human lung showed a medium to strong immunostaining signal for TRPC5. This includes pleura, subpleural connective tissue, pulmonary arteries and veins, bronchioles, alveolar septa, type 1 and 2 pneumocytes, and alveolar macrophages (Fig. 2).

All samples showed significant differences compared to the negative control (Fig. 2A). It can therefore be assumed that the TRPC5-protein is ubiquitously distributed in human lung tissue. Fig. 2 displays representative images of important histological features.

We also compared the differences in anti-TRPC5 staining signal between the male and the female donors, while excluding the male donor, who died of pneumonia (donor 2). Two of the three remaining male donors presented a medium staining signal (Fig. 2B and H), while one showed a strong staining signal (Fig. 2F). The tissue from both female donors presented a medium staining signal (Fig. 2E and G).

The comparison between the tissue from the male donor (donor 2) who died of pneumonia, and the other five donors without pneumonia revealed the following: The tissue from donor 2 presented a strong staining signal (Fig. 2C). In contrast, only one of the five donors without pneumonia presented a strong staining signal (Fig. 2F), while the other four presented a medium staining signal (Fig. 2B, E, G, and H).

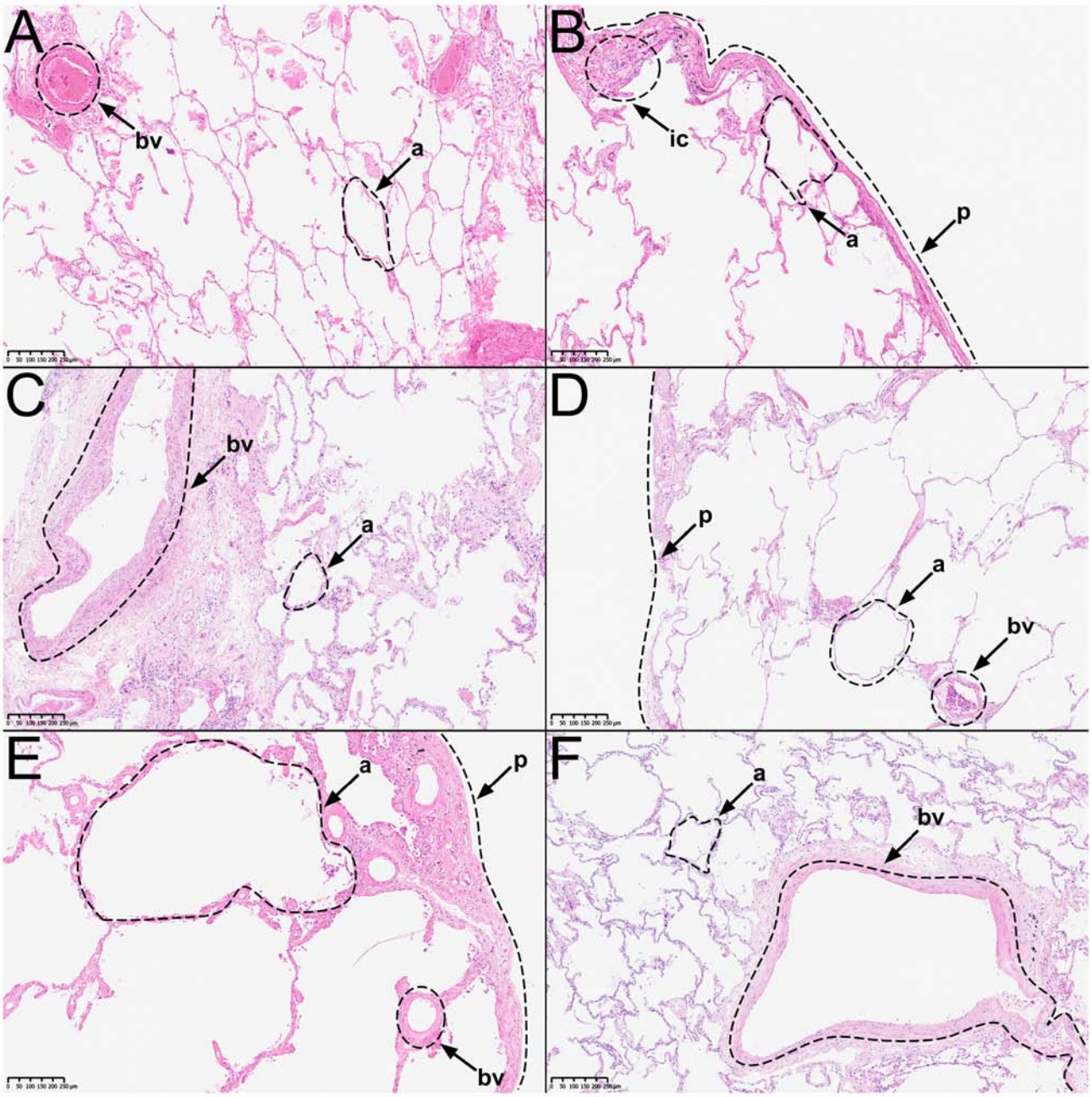

Figure 1. Representative hematoxylin & eosin staining. (A) Tissue from donor 1, (B) tissue from donor 2, (C) tissue from donor 3, (D) tissue from donor 4, (E) tissue from donor 5 and (F) tissue from donor 6. A, alveolus; BV, blood vessel; IC, immune cells; P, pleura.

Discussion

The anti-TRPC5 antibody we employed has already been used previously for immunohistochemistry (25,26). Additionally, the antibody has been validated on TRPC5-knockout mice in a previous study (27). We therefore assume that the antibodies were sufficiently specific to be used in our experiments. In each staining run, we included negative controls of the lung and positive controls of the heart. To ensure that DAB was converted by the horseradish peroxidase of the antibody and not by the endogenous peroxidases of the examined tissue, we deactivated the endogenous peroxidases by treating the tissue with 3% H_2O_2 solution, as aforementioned.

Whilst TRPC5 has already been detected in a few cell types of the lung, most studies have used cell cultures, animal cells or simply investigated the expression of TRPC5 mRNA. To our best knowledge, our study is the first to report TRPC5 in human lung tissue using immunohistochemistry. However, our study design has certain methodological limitations; The average age of the body donors we used was 81.3 years. Each donor died of natural causes. One of the donors died of pneumonia, caused by a bacterial infection. For staining, we used ten samples obtained from six body donors, four of which were male and two female. On one side the sample collective was rather small, since body donor availability is often restricted. Therefore, further validating research is needed to enable a generalized statement about the distribution and expression of the TRPC5-protein in the human lung. On the other side, the donors were of advanced age and because of that, the tissue might be prone to pathophysiological changes. The quality of

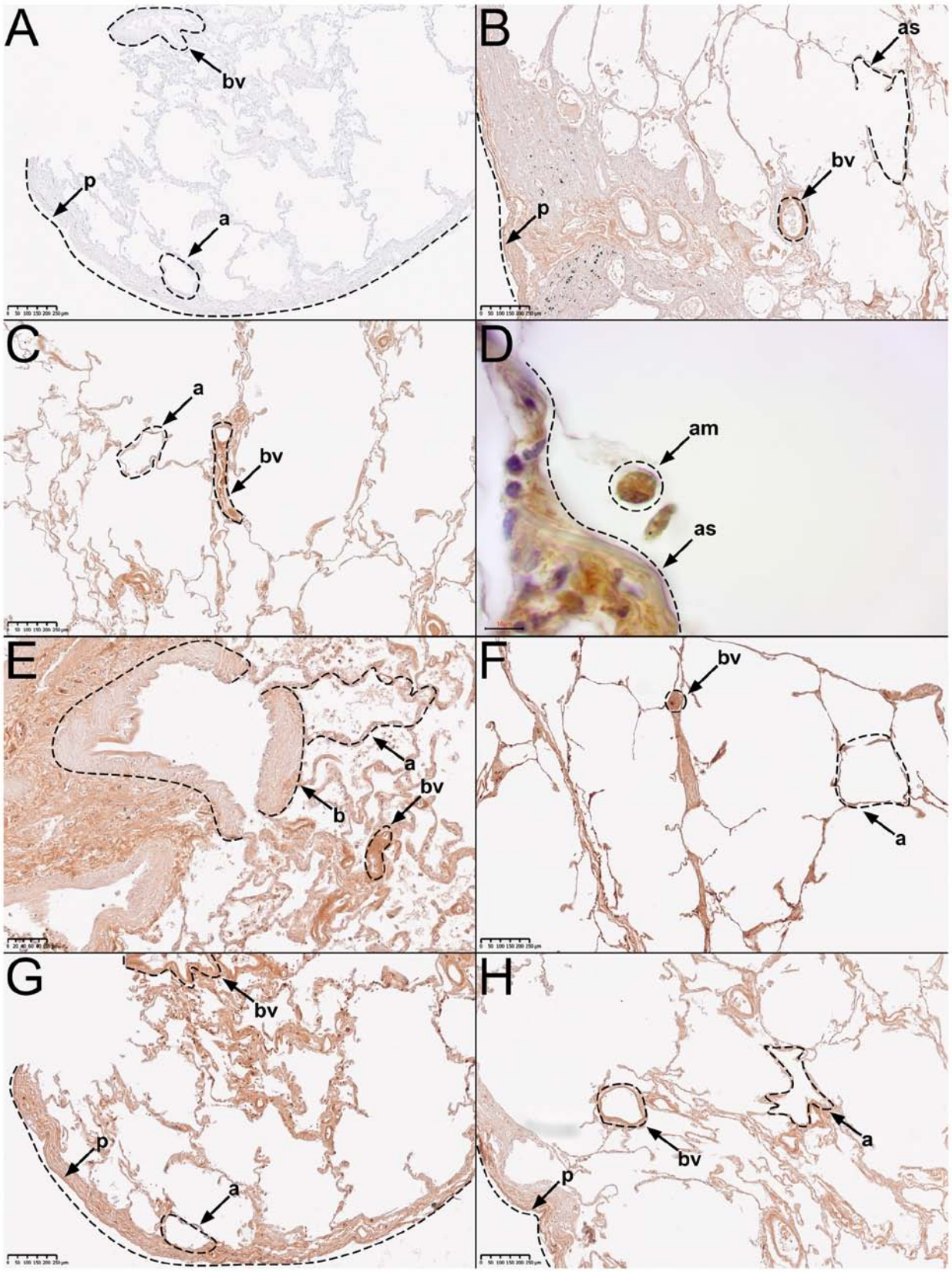

Figure 2. Representative immunohistochemical staining using a primary knockout-validated anti-TRPC5 antibody. (A) Negative control staining. (B) Tissue from body donor 1, tissue from donor 2 in (C) low and (D) high magnification, (E) tissue from donor 3, (F) tissue from donor 4, (G) tissue from donor 5, and (H) tissue from donor 6. AM, alveolar macrophage; A, alveolus; AS, alveolar septum; B, bronchiole; BV, blood vessel; P, pleura.

the samples was assessed beforehand, using H&E staining, and deemed acceptable. However, some more subtle pathophysiological changes cannot be detected by this method. Due to the advanced age of the body donors, the distribution of the TRPC5-protein in lungs of younger specimens remains elusive. For instance, expression levels of various proteins

are known to change throughout lifetime (28). Therefore, the distribution and expression intensity of TRPC5 may differ in younger individuals. Ultimately, the sole use of elderly body donors presents a methodological limitation of our study. In order to evaluate possible differences, further research should be conducted based on lung samples of younger specimens. However, this may prove complicated because of the rarity of young body donors, as most body donors die at an advanced age. Samples from younger, living donors could also be obtained from patients who have undergone biopsy or surgery for diagnostic or therapeutic purposes.

In our study we did not apply any exclusion criteria to the body donors. This poses a limitation to our study, as lung diseases could present confounding variables.

As mentioned above, we also compared the staining signals from lung tissues between male and female specimens. While one of the sections from the male donors presented a strong staining signal, and none of the female sections did, we cannot make a statement about the differences between male and female lung tissue in terms of TRPC5 immunostaining levels. Our sample size is too small and unbalanced in terms of sex distribution. For the comparison between the tissue of the male donor (donor 2) who died of pneumonia and the other five donors without pneumonia similar circumstances applied. While the tissue from donor 2 shows a strong staining signal, the sample of donor 4 also presents a strong staining signal. The sample size is too small to draw conclusions about differences in TRPC5 immunostaining levels. In addition, an infiltration of inflammatory cells in the lung tissue of donor 2 could cause changes to the staining signal due to the superimposition of positively and negatively stained cells. Therefore, staining signals could be misinterpreted. However, we found only slight signs of infiltration (Fig. 1B), and in most segments of the immunostained samples, no pneumonic infiltration is visible (Fig. 2C).

As mentioned above, we also stained all samples obtained from donor 5 to evaluate differences between extraction sites. Yet, we were unable to detect any differences. Similarly, we compared the sample from the central hilus-near extraction site of donors 4 and 5 with the peripheral lung samples. Again, no differences were detected. However, these findings are only preliminary. Due to the small sample size, we cannot make any statement about the difference in anti-TRPC5 staining signals between extraction sites or the difference between central and peripheral regions of the lung.

The fact that we only used immunostaining to detect the TRPC5-protein in the human lung is a restriction of our study. Further assays should be performed to solidify the evidence that TRPC5 is widely distributed in the human lung. This would also enable supportive investigation of possible differences in TRPC5-expression between donors with different characteristics, such as sex and age or between healthy and diseased tissue.

Another factor that needs to be discussed are the possible postmortem changes in protein levels and histological structures. In the present study, a major factor that contributes to unwanted postmortem changes is the time between the onset of death and the beginning of the preservation process. The start of preservation can be delayed by various factors like long transport time, delayed determination of death, or by official administrative matters. In our case, there were 1-4 days between the onset of death and the beginning of the preservation process. In a previous study, Cocariu *et al* (29) examined the correlation of refrigeration time and autolytic histological changes in lung tissue. They concluded that the deterioration of histological structures correlates with the time spent in the refrigeration unit (29). Protein levels could also be lowered due to autolysis. However, this process can be slowed down by lower temperatures (30). It is therefore essential that body donors are processed quickly after death. If this is not possible, the donor should be stored in a refrigeration unit until preservation.

To our knowledge, we were the first to detect the TRPC5-protein in human alveolar macrophages, by using immunohistochemistry. As mentioned in the introduction, Tao *et al* (14) showed that TRPC5 inhibits macrophage polarization into a proinflammatory phenotype and Pereira *et al* (15) suggested that TRPC5 in macrophages may play a protective role in sepsis. Thus, TRPC5 appears to be important for macrophage function. Alveolar macrophages are major players in the innate immune response and are critical for the proper maintenance of lung homeostasis (14,31). The topic of TRPC5 in alveolar macrophages may therefore be a promising area of research to better understand inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) (32).

To further solidify the evidence that TRPC5 is expressed in human alveolar macrophages, other experiments, such as RT-PCR to detect TRPC5 mRNA or Western blot to directly detect the TRPC5-protein could be performed.

Considering previous studies on the physiological function of TRPC5, a dysregulation of the channel may contribute to the pathogenesis of several lung diseases. These studies showed that disturbed cellular Ca^{2+}-homeostasis in airway smooth muscle may be due to dysregulation of TRPC5 (7,10,33,34). In smooth muscle cells, dysregulation of cellular Ca^{2+}-homeostasis is associated with bronchial asthma, pulmonary hypertension, and COPD (6,7,35). Moreover, the study by Chen *et al* (36) pointed out that impaired TRPC5-expression may contribute to the proliferation of pulmonary artery smooth muscle cells. Therefore, TRPC5 may become a target for pharmacological intervention in such conditions (6,37).

In this regard, TRPC5 has already been identified as a target for multiple pharmacological agents such as clemizole hydrochloride, GFB-887 or AC1903 (37-39). It is likely that other compounds will follow. Some of them may even be considered as candidates for the treatment of diseases that involve TRPC5-dysregulation (37). Interestingly, the TRPC5-inhibitor GFB-887 has already entered clinical trials, in patients with focal segmental glomerulosclerosis, treatment-resistant minimal change disease, and diabetic nephropathy (39).

As mentioned in the introduction, dysregulation of TRPC5 may be involved in the promotion of chemoresistance, proliferation, and invasion of cancer cells. It may be interesting to further investigate the role of TRPC5 in lung cancer. In that regard, TRPC5 could also present a drug target in cancer cells (16-18).

In conclusion, it can be stated that TRPC5 is likely to play an important role in pulmonary cell function, particularly in cellular cation homeostasis. Dysregulation of cellular cation

homeostasis is associated with various lung diseases such as bronchial asthma and pulmonary hypertension. Previous studies have already implicated dysregulation of TRPC5 as part of the pathogenesis of such lung diseases (6,7,10,33-35). Our study suggests that TRPC5 is widely distributed throughout the human lung. Pharmacological agents targeting TRPC5 have already been discovered. Therefore, TRPC5 may present a new target for the treatment of various lung diseases (37-39). Since the importance of TRPC5 in such pharmacological interventions is highly speculative at present, it is clear that the properties of TRPC5 and its involvement in lung physiology and pathophysiology should be further investigated.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

TT and FU planned and conducted the study. FU performed the experiments. FU, FF, TT, CMD and CNE evaluated and interpreted the data. FU and TT confirm the authenticity of all the raw data. FU wrote the first draft of the manuscript. TT, CMD and CNE reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All body donors gave written consent during their life-time for their bodies and tissues to be used for scientific research. In addition, the conducted study was approved by the ethical commission of the Saarland Medical Association (Ärztekammer des Saarlandes) under the approval number 163/20. The responsibility of the above mentioned committee for the conducted study was approved by the Dean of the Saarland University and this document was deposited at the publisher (Spandidos Publications).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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