

MAJOR TECHNICAL ADVANCES

Multiresolution 3D Optical Mapping of Immune Cell Infiltrates in Mouse Asthmatic Lung

Yi-Chien Wu¹, Hyung-Geun Moon², Vytas P. Bindokas³, Evan H. Phillips¹, Gye Young Park^{2,4}, and Steve Seung-Young Lee¹

¹Department of Pharmaceutical Sciences and ²Division of Pulmonary, Critical Care, Sleep and Allergy, Department of Medicine, University of Illinois Chicago, Chicago, Illinois; ³Integrated Light Microscopy Facility, The University of Chicago, Chicago, Illinois; and ⁴Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois

ORCID IDs: 0000-0002-2877-4476 (Y.-C.W.); 0000-0003-1349-3759 (S.S.-Y.L.).

Abstract

Asthma is a chronic inflammatory airway disease driven by various infiltrating immune cell types into the lung. Optical microscopy has been used to study immune infiltrates in asthmatic lungs. Confocal laser scanning microscopy (CLSM) identifies the phenotypes and locations of individual immune cells in lung tissue sections by employing high-magnification objectives and multiplex immunofluorescence staining. In contrast, light-sheet fluorescence microscopy (LSFM) can visualize the macroscopic and mesoscopic architecture of whole-mount lung tissues in three dimensions (3D) by adopting an optical tissue-clearing method. Despite each microscopy method producing image data with unique resolution from a tissue sample, CLSM and LSFM have not been applied together because

of different tissue-preparation procedures. Here, we introduce a new approach combining LSFM and CLSM into a sequential imaging pipeline. We built a new optical tissue clearing workflow in which the immersion clearing agent can be switched from an organic solvent to an aqueous sugar solution for sequential 3D LSFM and CLSM of mouse lungs. This sequential combination microscopy offered quantitative 3D spatial analyses of the distribution of immune infiltrates in the same mouse asthmatic lung tissue at the organ, tissue, and cell levels. These results show that our method facilitates multiresolution 3D fluorescence microscopy as a new imaging approach providing comprehensive spatial information for a better understanding of inflammatory lung diseases.

Keywords: multi-resolution 3D microscopy; optical tissue clearing; immune infiltrates; asthma

Asthma is a chronic inflammatory disorder of the lung that affects approximately 8% of the U.S. population (1). Despite advances in therapeutics, the underlying biological and immunological mechanisms of asthma have not yet been fully elucidated. Our current understanding is that the innate and adaptive immune systems are involved in the

initiation and progression of asthma (2). The innate and adaptive immune factors mediate asthmatic reactions within the complex three-dimensional (3D) architecture of the lung, which is characterized by having multiple distinct lobes and a branching airway network referred to as the tracheobronchial tree (3). As such, in an

effort to understand the pathogenesis of asthma and develop effective treatments for asthma, it is critical to achieve multiscale 3D spatial lung immune profiles, including information on pulmonary anatomy, the pathological tissue site, and the distribution and phenotypes of individual immune cells.

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Correspondence and requests for reprints should be addressed to Steve Seung-Young Lee, Ph.D., Department of Pharmaceutical Sciences, University of Illinois Chicago, 833 South Wood Street, Chicago, IL 60612. E-mail: ssylee@uic.edu.

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Clinical Relevance

Our switchable tissue-clearing method enabled multiresolution three-dimensional optical microscopy and three-dimensional spatial mapping of immune cell infiltrates in the mouse asthmatic lung from the scale of the whole organ to the single-cell scale. This method has great potential for comprehensive spatial profiling of various cell types and their interactions in human and preclinical animal lung tissues, not only for discovery of new biomarkers but also for evaluation of drug candidates for effective asthma therapy.

Confocal laser scanning microscopy (CLSM) is a well-validated imaging method in asthma research (4). Blocking of out-of-focus light and the use of high-magnification objectives in CLSM permit single-cell imaging in lung tissue samples. Together with multicolor immunofluorescence (IF) staining, CLSM enables visualization of various immune cell types and their interactions in the tissue microenvironment (5). In addition, applying an aqueous-based optical tissue-clearing method increases the imaging depth of CLSM, which can produce cellular-resolution 3D maps of multiple biomarkers throughout a half-millimeter tissue (5, 6). Although CLSM can provide image data over a large field of view (FOV) from a tissue section at a high resolution, its point-scanning approach requires extended imaging time and intensive labor for the stitching and reconstruction of big image data, which is impractical for a large tissue sample such as the whole mouse lung. On the contrary, LSFM offers fast 3D imaging of a large tissue sample by generating thin sheets of illumination light and scanning through an entire tissue specimen (6, 7). A commercial LSFM using a low magnification and long working distance objective (4× or 12×) can generate macroscopic and mesoscopic 3D visualization of the structural organization and morphology of functional tissue units in whole mouse organs (8, 9). Thus, it is expected that applying CLSM and LSFM for the same lung tissue would produce

comprehensive spatial information across the whole-organ, tissue, and cellular levels by resolving the conventional tradeoff in optical microscopy between image resolution and FOV. Specifically, in the case of having only a limited number of samples or a small lung tissue sample for an imaging assay, this combination microscopy approach could help maximize collection of multiresolution 3D image data and critical spatial information from each precious preclinical and clinical asthma research sample.

Recent progress in optical tissue-clearing methods allows CLSM and LSFM to produce 3D images of tissue samples (10). Simple incubation in a series of immersion solutions turns opaque tissues into transparent and light-permitting specimens by uniformizing the refractive index (RI) of cellular and noncellular components (e.g., phospholipids, extracellular matrix, proteins, and DNA) in tissue (11, 12). For 3D LSFM, less toxic or nontoxic organic solvents such as dibenzyl ether (DBE) and ethyl cinnamate (ECi) have been generally applied to acquire greater transparency in large-volume tissues and whole mouse organs after alcohol-mediated tissue dehydration (13–15). Organic solvent-based tissue clearing methods are compatible with LSFM systems equipped with a solvent and specimen reservoir, immersion objectives, and dipping caps. Because a conventional CLSM system does not use dipping caps on objectives and has an open sample stage for tissue slides, aqueous solution-based tissue-clearing techniques using highly concentrated sugar (e.g., fructose [5] and sucrose [16]) solutions are more practical for high-resolution 3D CLSM imaging. In turn, these two tissue-clearing methods with different immersion chemicals and procedures make concurrent use of CLSM and LSFM incompatible for 3D imaging of a single tissue sample to generate multiscale 3D image data.

To address this challenge, we present here a novel workflow that sequentially switches optical tissue clearing of mouse lung tissues immersed in organic solvents (DBE and ECi) to an aqueous solution (D-fructose). This workflow allows sequential LSFM and CLSM for multiresolution 3D imaging of lung tissues from a mouse model of allergic asthma. With a reversal of the organic solvent-based tissue-clearing procedure after LSFM, the same lung tissue can proceed to aqueous-based tissue clearing and 3D CLSM imaging. Our workflow was validated not only for preserving immune cell-surface

markers (e.g., the cluster of differentiation) but also for maintaining IF signals in mouse lung tissues. Throughout our workflow, LSFM revealed immune infiltrates within a whole mouse lung, and then CLSM localized CD45⁺ CD3⁺ T lymphocytes in the same lung tissue at cellular resolution. Quantitative 3D spatial analyses revealed the regional preference of T lymphocytes and their distance from pulmonary blood vessels and airways. Distinct numbers and subtypes of immune cells were also found in normal and asthmatic lungs with a variety of CD45 and CD3 expression patterns. These results demonstrate that switching optical tissue-clearing agents from organic solvents to aqueous sugar solutions enables the use of both LSFM and CLSM as a new imaging assay tool to achieve comprehensive 3D spatial information of lung tissues for asthma research.

Methods

Mouse Asthma Model

The asthma mouse lung was harvested from a 7-week-old female C57BL/6 mouse with asthma induced by triple allergens of dust mite, ragweed, and *Aspergillus*. The normal lung was harvested from a healthy 7-week-old female C57BL/6 mouse without any vehicle treatment. Further details are included in the data supplement.

Collection, Decolorization, and IF Staining of Mouse Lungs

All steps involved gentle shaking. Mouse lungs were fixed in 2% paraformaldehyde solution (in PBS solution) for 30 minutes at room temperature. Then, the lungs were decolorized in red blood cell (RBC) lysis buffer (cat. no. 11814389001; Roche/Sigma-Aldrich) for 1 day at 4 °C. IF staining of the entire lungs with a 1:40 vol/vol ratio of DyL550-CD45 antibody was performed in staining buffer (PBS solution, pH 7.4, with 10 mg/ml BSA and 0.3% Triton X-100) for 4 days at 4 °C. Further details are included in the data supplement.

Organic Solvent-Based Tissue Clearing

Organic solvent-based tissue clearing includes dehydration with ethanol (EtOH) solutions and RI matching with DBE (cat. no. 108014; Sigma-Aldrich) and ECi (cat. no. 112372; Sigma-Aldrich). The pH of the solutions was adjusted to 9.0 or higher

with triethylamine. A total of 30 μ l of 1-thioglycerol (TG) was added per 10 ml of solution as an antioxidant. Tissue clearing was performed at 4 °C with gentle shaking except that ECI clearing was performed at room temperature. Further details are included in the data supplement.

LSFM

LSFM image acquisition was performed on a light-sheet fluorescence microscope (Ultramicroscope II; LaVision BioTec, now part of Miltenyi Biotec) in the Integrated Light Microscopy Facility at the University of Chicago.

Reverse Tissue Clearing and Vibratome Macrosectioning

The same mouse lungs were subjected to reverse organic solvent-based tissue clearing. The rehydrated lungs were then embedded in 2% agarose gel and sectioned at 1- or 1.5-mm thickness with a vibratome (VT1200S; Leica). Further details are provided in the data supplement.

Additional Multiplex IF Staining and Aqueous Sugar-Based Tissue Clearing

Lung macrosections were further stained with DyL488 anti- α smooth muscle actin (α SMA) and Alexa 594 anti-CD3 antibodies, washed in PBS solution, and optically cleared in an ascending gradient of D-fructose solutions with gentle shaking. TG was added to the D-fructose solutions as an antioxidant. Further details are included in the data supplement.

CLSM

Confocal images were acquired using a Leica TCS SP8 laser scanning confocal microscope in the Integrated Light Microscopy Facility at the University of Chicago. Further details are included in the data supplement.

Image Data Processing

Image processing, display, and quantitative measurements were performed in Fiji (<https://fiji.sc/Fiji>) and OXFORD Imaris (<https://imaris.oxinst.com>). Measurement data are presented as mean $\pm$ SD, and statistics were run using two-way ANOVA or two-sample *t* tests in Origin 2022 (<https://www.originlab.com/>). Further details are included in the data supplement.

Results

Workflow for Multiresolution 3D Fluorescence Microscopy of Mouse Lung Tissues

We created a robust workflow for processing lung tissues involving organic solvent and aqueous solution-based optical tissue clearing methods (Figure E1A in the data supplement). The workflow allowed for 3D LSFM and CLSM of the same lung tissue sequentially, enabling multiresolution 3D fluorescence microscopy. To model an imaging assay of human asthmatic lung tissues, we have validated our approach for examining immune infiltrates in the lung tissue from a mouse model of allergenic asthma (17). Sufficient tissue transparency is the prerequisite for successful 3D LSFM imaging. To prevent light scattering and absorption by heme in RBCs during LSFM, we performed transcardiac perfusion with PBS solution before harvesting the lung tissues. We further removed heme in the collected lung tissues using RBC lysis buffer (Figures E1B and E2). Next, we stained the tissues with DyLight 550 anti-CD45 antibody for 4 days. This incubation time allowed for homogenous IF labeling of hematopoietic immune cells in the whole lungs. To make the lung tissues miscible in an organic solvent for RI matching-mediated optical clearing, prerenoval of water in the tissues is necessary. Moreover, it is important to preserve protein antigens in lung tissues during organic solvent-based clearing for the sequential IF staining and CLSM imaging in the same lung tissue afterward. We tested different organic solvents, including EtOH, methanol, and tetrahydrofuran, for tissue dehydration, and found that EtOH-based dehydration maintains integrity and antigen immunogenicity of various immune cell types, such as B220 (B cells), F4/80 (macrophages), CD11c (dendritic cells), and CD3 (T cells), for IF detection (Figure E3). Thus, we incubated the lungs sequentially in an increasing gradient of EtOH solutions that contained an antioxidant, TG, for tissue dehydration. The dehydrated lung tissues were then sequentially incubated in DBE and ECI solvents for the RI matching. To keep the protein antigens and fluorescence signal stable in the lung tissues, the pH of all ethanol solutions as well as DBE and ECI solvents was adjusted to 9.0, and TG was also added to the solvents. This preprocessing and organic solvent-based optical clearing

permitted 3D LSFM imaging of whole mouse lungs at macroscopic and mesoscopic resolution.

Although LSFM offered organ-level 3D images of immune infiltrates in whole mouse lungs, image resolution was insufficient for clear visualization of individual cells and their functional protein expression (18). Thus, we compensated for this low spatial resolution issue by adding CLSM to the imaging pipeline. After LSFM, mouse lungs were subjected to the reverse organic solvent-based optical tissue clearing (see Figure E1B, bottom row). Transferring from ECI to EtOH converted the lung tissues from transparent back to opaque. After rehydration with a lower EtOH concentration and then PBS solution, lungs were embedded in agarose gels and sectioned at 1- or 1.5-mm thickness. After additional IF staining for other cellular markers, lung macrosections were incubated in a series of D-fructose solutions for aqueous optical tissue clearing. Our previous reports showed that simple immersion of tissue macrosections in high-concentration D-fructose solutions enabled the achievement of adequate tissue transparency for high-resolution 3D CLSM imaging without noticeable changes in tissue volume and IF intensity (5, 6). The 3D confocal microscopy of cross-sectioned areas of lung tissues enabled *in situ* analysis of immune infiltrates at cellular resolution. Thus, through this multiresolution 3D fluorescence microscopy workflow, we obtained comprehensive organ-, tissue-, and cellular-level image data from representative normal and representative asthmatic mouse lungs (*n* = 1 per group). The multilevel lung image data are described in the following sections.

Organ-Scale 3D Visualization and Quantification of Immune Cell Infiltrates in the Asthmatic Mouse Lung Using LSFM

Single representative mouse lungs from normal and asthmatic groups were selected to acquire 3D images by LSFM after organic solvent-based optical tissue clearing (Figure 1 and Videos E1 and E2). LSFM using an Ultramicroscope II (LaVision BioTec, now part of Miltenyi Biotec) is capable of imaging a depth of field of more than 5.6 mm without the loss of image contrast in the middle of the lung tissues (Figure E4 and Video E3). Light-sheet scanning of autofluorescence signal in mouse lungs enabled delineation of the branching airway network.

This nondestructive 3D visualization depicted a complete tracheobronchial tree in the lung. Strong fluorescence intensity of CD45⁺ antibody labeling hematopoietic immune cells was observed in a broad area of the asthmatic lung (Figure 1A). The tissue volume in which CD45⁺ hematopoietic immune cells accumulated in the asthmatic lung was approximately 25 times larger than that in the normal lung (Figure 1B). Higher magnification and virtual orthoslice images of the asthmatic lung showed that CD45⁺ hematopoietic immune cells were mainly located along the airways, with few of the cells in the lung parenchyma (Figures 1C and 1D and E5). To analyze the infiltration of CD45⁺ hematopoietic immune cells in different anatomical regions of the lung, we segmented the 3D image of the representative asthmatic lung into five

lobes and the trachea (Figures 2A and 2B and Video E4). Quantitative analysis of the regional distribution of CD45⁺ hematopoietic immune cells in the asthmatic lung showed that most CD45⁺ cells were predominantly present in the left (45%), superior (23%), and inferior (25%) lobes (Figure 2C). Even after considering the volume difference of each compartment, this pattern of cell distribution was not changed (Figure 2D). We then virtually extracted two subsegmental bronchi (*i* and *ii*; see Figure 3) with clear autofluorescence signals in the image data of the representative asthmatic lung (Figure 2E) and performed pseudocolor distance mapping (Figures 2F and 2G). We determined the distance profiles of hematopoietic immune cells surrounding each bronchus (Figures 2H and 2I and Videos

E5 and E6). Most hematopoietic immune cells (97% of hematopoietic immune cells surrounding bronchus *i* and 100% of hematopoietic immune cells surrounding bronchus *ii*) were located within a 1,000- μ m distance from the surface of the nearest bronchus. The average distance of hematopoietic immune cells from bronchi *i* and *ii* were 357.54 μ m and 224.32 μ m, respectively. Interestingly, approximately 15% of the hematopoietic immune cells showed zero or negative values in the distance profiles, which might indicate the migration of hematopoietic immune cells into the inflamed airway lumen through the smooth muscle wall (19, 20). These results demonstrate that organ-level LSFM imaging offers quantitative 3D spatial mapping of immune infiltrates in the whole mouse lung for asthma research.

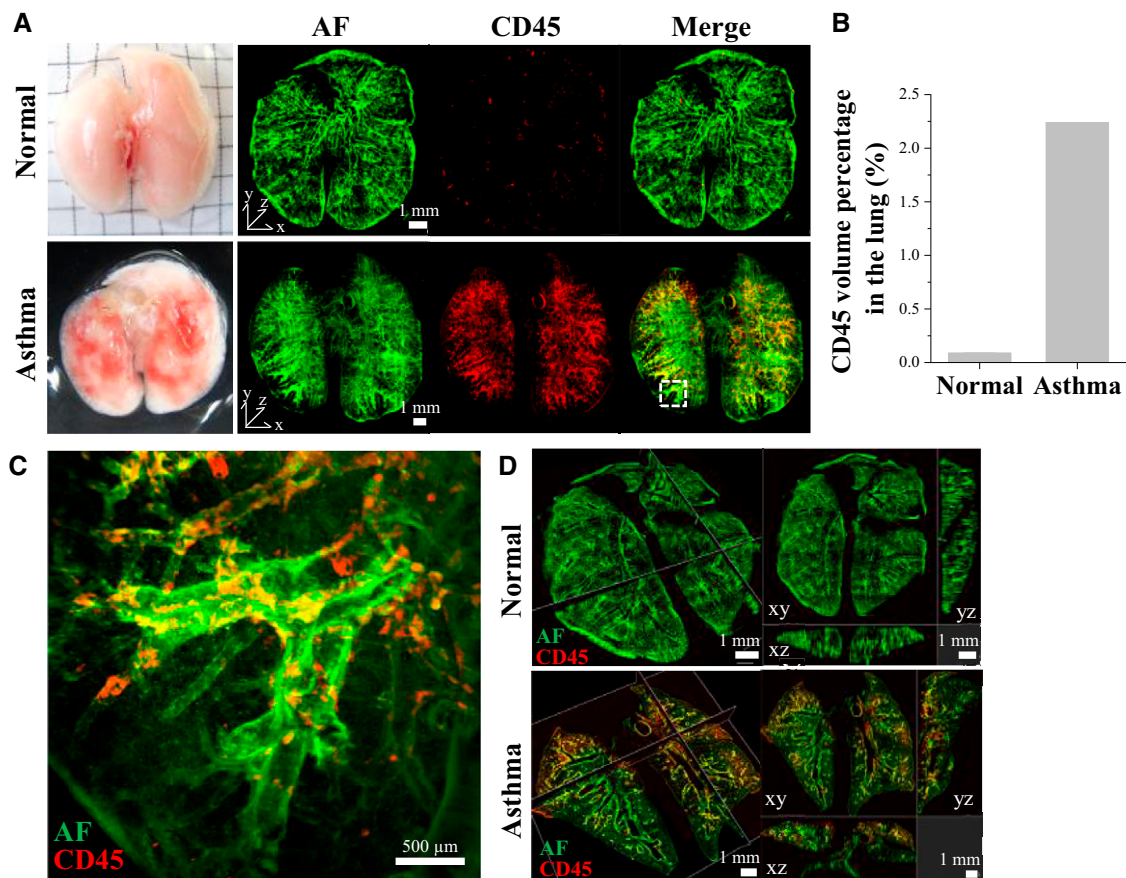


Figure 1. Three-dimensional (3D) light-sheet fluorescence microscopy (LSFM) images of immune infiltrates in whole mouse lungs. (A) Photographs (left) of two representative lungs (one normal and one asthmatic) before processing and 3D-rendered LSFM images of the normal and asthmatic mouse lungs. Autofluorescence (green) and CD45 (red) signals mark the lung airways and hematopoietic immune cells, respectively. Scale bars, 1 mm. (B) Quantification of volume percentages of CD45⁺ hematopoietic immune cells in the representative lungs shown in A. (C) Magnified image of the marked area (white dotted box) in A (bottom right in the asthmatic lung images) shows CD45⁺ hematopoietic immune cells along the airways. Scale bar, 500 μ m. (D) Orthogonal projections of the 3D lung images in A onto xy, xz, and yz planes. Scale bars, 1 mm. AF = autofluorescence.

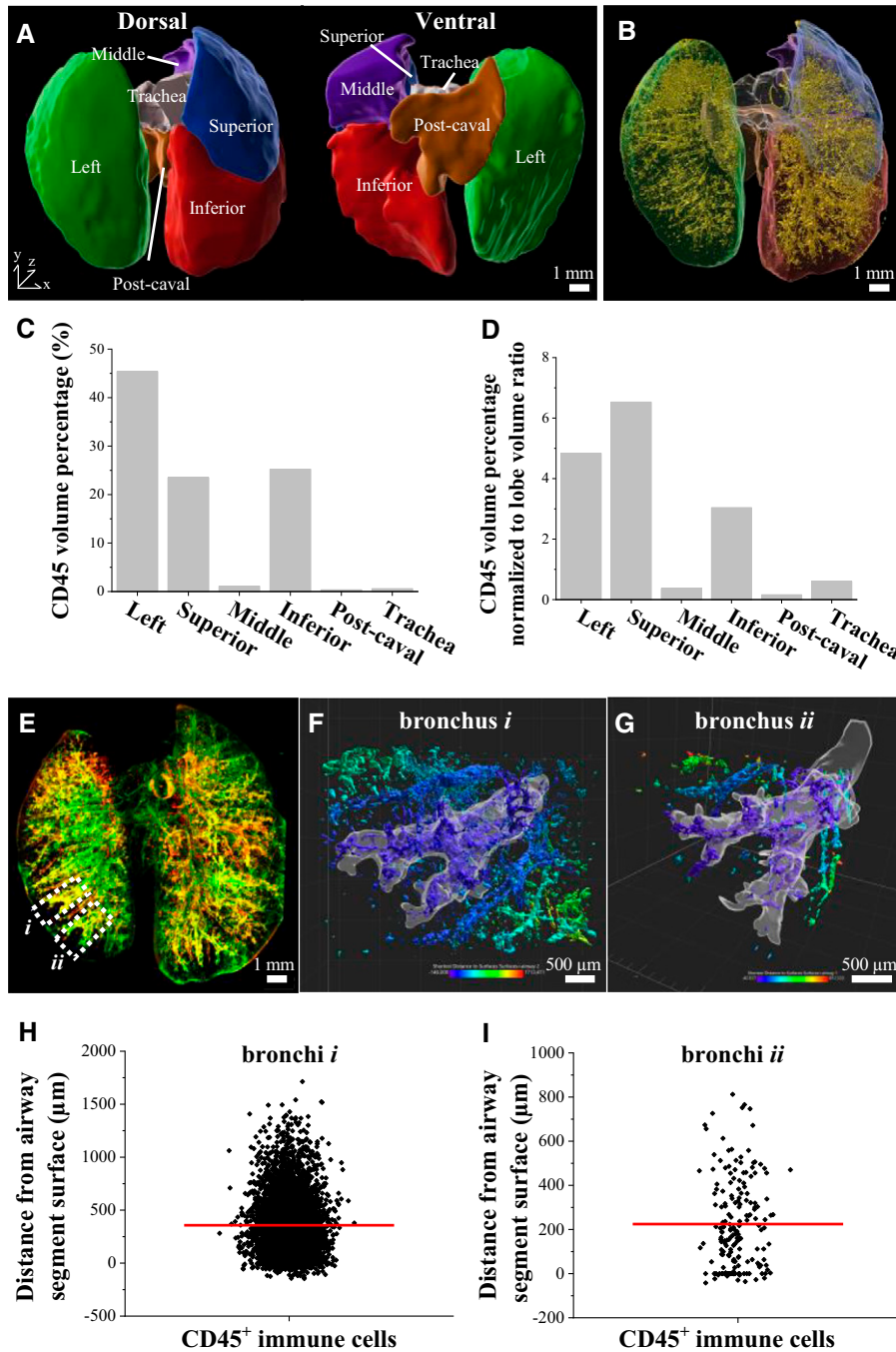


Figure 2. Regional distribution of immune infiltrates in asthmatic lung. (A) A 3D surface rendered of the left (green), superior (blue), middle (magenta), inferior (red), and postcaval (brown) lobes and the trachea (gray) segmented from the LSFM images of the asthmatic lung in Figure 1A. Views are of the dorsal (left) and ventral (right) sides. Scale bar, 1 mm. (B) A 3D visualization of segmented CD45⁺ hematopoietic immune cells (yellow) overlapped with a transparent-rendered image of the asthmatic lung compartments shown in A. Scale bar, 1 mm. (C) The volume percentage of CD45⁺ hematopoietic immune cells in the lung lobes and trachea of the representative asthmatic lungs in B. (D) Normalization of volume percentage of CD45⁺ hematopoietic immune cells in the lung lobes and trachea. The percentage values in C were divided by the volume ratios of the lung compartments. (E) Representative subsegmental bronchi (*i* and *ii*) selected from the representative asthmatic lungs in Figure 1A. Scale bar, 1 mm. (F and G) Pseudocolor distance maps of CD45⁺ hematopoietic immune cell segments from the surfaces of bronchi *i* (F) and *ii* (G). The color scale bars in F and G represent distances over the ranges of $-146\ \mu\text{m}$ (blue) to $1,713.471\ \mu\text{m}$ (red) and $-42.037\ \mu\text{m}$ (blue) to $812.333\ \mu\text{m}$ (red), respectively. Scale bars, $500\ \mu\text{m}$. (H and I) The 3D distance profiles of CD45⁺ hematopoietic immune cells away from bronchi *i* (H) and *ii* (I). The red line marks the average distance of CD45⁺ hematopoietic immune cells from bronchi *i* and *ii*.

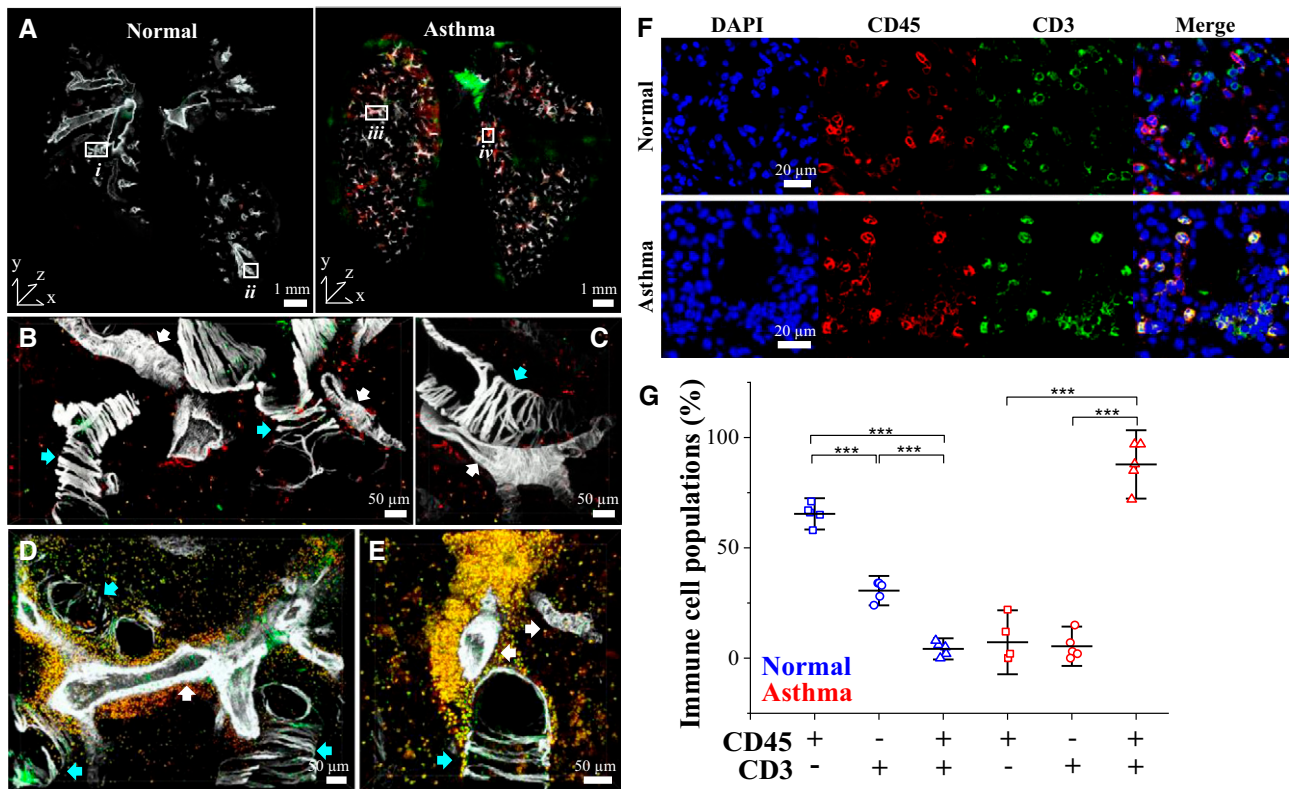


Figure 3. Tissue-scale 3D confocal laser scanning microscopy (CLSM) images of immune infiltrates and cell-resolution imaging assay of immune cell populations in normal and asthmatic mouse lungs. (A) The 3D rendered CLSM images of a normal (left) and an asthmatic (right) lung macrosection, each from the representative lungs shown in Figure 1. The lung macrosections were stained with α -smooth muscle actin (gray), CD45 (red), and CD3 (green) antibodies. Scale bars, 1 mm. (B and C) Magnified 3D images of areas *i* (B) and *ii* (C) are marked (white square) in the normal lung image in A (left). White and cyan arrows point to the pulmonary arteries and airway branches, respectively. Scale bars, 50 μ m. (D and E) Magnified 3D images of areas *iii* (D) and *iv* (E) are marked (white square) in the asthmatic lung image in A (right). Scale bars, 50 μ m. (F) Cell-level CLSM images of CD45⁺ (red) and CD3⁺ (green) immune cells in the representative normal and asthmatic lung macrosections in A. DAPI signal (blue) indicates individual cell nuclei in the images. Scale bars, 20 μ m. (G) Quantification of CD45 and CD3 immune cell populations by cell enumeration in five different areas of normal and asthmatic lung macrosections, respectively. Values are displayed as mean $\pm$ SD, and statistics were run using two-way ANOVA (** $P \leq 0.001$).

Tissue-Scale 3D Spatial Mapping of Immune Infiltrates in Mouse Asthmatic Lung Using CLSM

To obtain images with higher spatial resolution after LSM acquisition, the same representative normal and asthmatic lungs underwent reverse processing of organic solvent-based optical tissue clearing and dehydration. Then, the lung tissues were cut into macrosections, and one middle macrosection of each normal and asthmatic lung was selected for further processing and CLSM imaging. To determine hematopoietic immune cell subtypes and visualize their locations along with 3D microanatomy of lungs, such as pulmonary arteries and airways, lung macrosections (with preexisting DyLight 550 anti-CD45 antibody staining) were costained with DAPI, DyLight 488- α SMA, and Alexa 594-anti-CD3e (or CD3) antibodies. The tissue macrosections

were then incubated in D-fructose aqueous solutions to yield sufficient transparency for high-resolution 3D CLSM imaging (Figure 3A). We selected regions *i* and *ii* in the normal lung (Figure 3A, left) and regions *iii* and *iv* in the asthma lung (Figure 3A, right), where airways and arteries are in close proximity for tissue-scale imaging (Figures 3B–3E for regions *i*–*iv*, respectively). Although it was difficult to clearly localize immune cells in the normal lung by LSM (Figures 1A and 1D), CLSM using a 40 $\times$ oil objective enabled us to visualize individual CD45⁺ hematopoietic immune cells and CD3⁺ lymphocytes located sporadically around the normal airway (Figures 3B and 3C). As seen with LSM image data, higher intensity and broadly distributed fluorescence signal of immune cell infiltrates was observed in the asthmatic lung compared with the normal lung (Figures 3D

and 3E and E6A). Furthermore, the unique expression patterns of α SMA permitted the differentiation of pulmonary arteries from airways in lung tissues (21, 22). α SMA⁺ myofibroblasts on the bronchial airway (AW) wall were oriented perpendicular to the course of the airway lumen and exhibited regular gaps between them (Figures 3B–3E, cyan arrows), whereas α SMA⁺ smooth muscle cells on the pulmonary artery (PA) wall formed a continuous tight layer (Figures 3B–3E, white arrows). Based on these distinct patterns of α SMA expression, we segmented PAs and AWs in the 3D CLSM images of the representative asthmatic lung macrosection and characterized the distances of CD45⁺ hematopoietic immune cells from the surfaces of the closest segmented AWs and PAs. Importantly, we noticed that most CD45⁺ hematopoietic immune cells were localized significantly

closer to the PAs (average distance, 31.72 μm) than the AWs (average distance, 85.46 μm) (see Figures E6D and E6E). In contrast, this phenomenon was not observed in a normal lung macrosection, which revealed similar distances of immune cells from the AWs (average distance, 91.47 μm) and the PAs (average distance, 71.86 μm) (see Figures E6B and E6C). Profiling of immune cell distance was quantified respectively on the regions where we found AW and PA nearby in the 3D images (Figures 3C and 3D) of the representative normal and asthmatic lung macrosections. These tissue-scale 3D image data produced by CLSM thus provide quantitative spatial information on immune cell infiltrates in the context of the microanatomical structure of the asthmatic lung.

CLSM

We could clearly localize individual immune cells in the same representative normal and asthmatic lung macrosections by CLSM imaging of CD45 and CD3 markers using a high-magnification 40 $\times$ oil objective (Figure 3F). CD45 and CD3 have generally been used as markers for hematopoietic immune cells and T lymphocytes, respectively (23, 24). We noticed that CD45 and CD3 expression patterns of immune cells in normal and asthmatic lungs were significantly different. The majority of immune cells in the normal lung expressed CD45 or CD3, whereas the main immune cell population in the asthmatic lung coexpressed both CD45 and CD3 (Figure 3F). Interestingly, in the asthmatic lung, many proliferating multinucleated immune cells with strong membrane and cytoplasmic CD45 and CD3 expression were observed. Quantitative analysis was performed in five randomly selected regions in the same normal and asthmatic lung macrosections. A total of 65% of the immune cells in the normal lung were CD45⁺CD3⁻, 31% were CD45⁻CD3⁺, and the remaining 4% were CD45⁺CD3⁺ (Figure 3G). In contrast, almost 90% of the immune cells in the asthmatic lung were CD45⁺CD3⁺ and the remaining 10% were CD45⁺CD3⁻ or CD45⁻CD3⁺. Our results, therefore, show that high-resolution multiplex fluorescence CLSM imaging of the same lung tissue enables *in situ* analysis of immune protein marker expression at the single-cell level, providing additional cell information.

Discussion

Asthma is a complex chronic inflammatory lung disease that is associated with various immune cell types (2, 25). Circulating innate and adaptive immune cells migrate into the lung by chemotaxis that is triggered by airway-resident and structural cells in response to inhaled allergens. Excessive immune-immune cell and immune-stromal cell interactions orchestrate aggressive allergic inflammation in the lung, eventually causing overproduction of mucus and dysfunction of airways (26). Thus, studying immune cell infiltration in the lung is critical not only for elucidating the immunopathology of asthma but also for developing effective therapies. Although advanced cellular and molecular assay methods such as multiplex flow cytometry and single-cell RNA sequencing can identify detailed phenotypes of individual immune cells in lung tissues (27, 28), spatial information is missing from such cell and transcriptome data. Traditional two-dimensional (2D) immunohistochemical assays of thin tissue sections have shown difficulty in accessing complex anatomical structures of the lung. Because the lung is one of the large-volume organs and has a complicated 3D anatomical structure consisting of five lobes and extensively branched airways (i.e., tracheobronchial tree), different regions of the lung constitute distinct tissue microenvironments that directly affect the infiltration and activation of immune cells in the development of asthma (29). Therefore, to investigate the underlying mechanisms of asthma and to avoid misinterpretation related to limited 2D image data, it is important to assess 3D spatial information of immune infiltrates and their functional or activated status along with the full anatomical lung structure.

Here, we report a methodology for multiresolution 3D fluorescence microscopy and its application to 3D mapping of immune infiltrates in the asthmatic mouse lung over the whole-organ to cellular scales. LSFM of whole mouse organs can provide macroscopic and mesoscopic resolution for 3D visualization of tissue and organ structures, which is enabled by organic solvent-based optical tissue clearing (8, 9). Although LSFM using the commercially available Ultramicroscope (initially developed by LaVision BioTec, now part of Miltenyi Biotec) has been applied to localize individual cells in

tissues, light-sheet scanning even with the highest-magnification objective (e.g., 4 $\times$, numerical aperture 0.3) in the system cannot provide sharp contrast and clear images of single cells (18). In contrast, conventional CLSM with a high-magnification objective (e.g., 40 $\times$ oil, numerical aperture 1.25) can provide cellular-resolution 3D image data from tissues after aqueous solution-mediated tissue clearing (30). However, with CLSM, there is a tradeoff in imaging speed and FOV for image resolution. To overcome these limitations and enable multiscale 3D imaging of mouse lung tissues, we developed a workflow of switching optical tissue-clearing methods. Through the workflow, lung tissue can be processed with DBE and ECi solvents and aqueous D-fructose solution in sequence to offer sufficient tissue transparency for 3D LSFM and CLSM imaging, respectively. In previous studies, reversing the tissue-clearing process after 3D microscopy has been shown to facilitate the acquisition of additional 2D spatial information from the same tissue sample through further histological processing and immunohistochemistry assays (31–33). However, to the best of our knowledge, the approach of reversing tissue clearing has yet to be applied to switching to another tissue-clearing procedure for sequential fluorescence microscopy to access multiresolution 3D image data from the same tissue sample.

Other challenges in 3D imaging of asthmatic mouse lungs include rendering the tissue sufficiently transparent while maintaining structural integrity and immunogenicity of protein antigens for IF detection of various immune cell types (10, 34). Transcardiac perfusion before tissue collection could not effectively remove RBCs in the patchy inflammation regions of asthmatic lungs (see Figure E2). Because heme in RBCs absorbs and scatters light, depletion of RBCs from the lung is critical for 3D LSFM imaging. To address this problem, we used an RBC lysis buffer that has generally been used to selectively remove RBCs in blood samples or cell suspensions from tissues before running flow cytometry. The use of RBC lysis buffer successfully eliminated residual RBCs and decolorized the asthmatic lung, achieving similar transparency to the normal lung after tissue clearing. To date, several organic solvents have demonstrated the potential for tissue dehydration and optical clearing for 3D LSFM imaging (8, 9). To demonstrate the compatibility of additional IF staining with immune cell markers for downstream

confocal microscopy, we explored the organic solvents that do not significantly reduce the immunogenicity of representative immune cell markers in the mouse lungs (see Figure E3). EtOH-mediated tissue dehydration enabled IF imaging of various immune cells in lung tissues by maintaining the structural integrity and immunogenicity of the membrane protein antigens, including CD3, CD11c, B220, and F4/80. However, a general problem of tissue shrinkage in tissue dehydration processing is still inevitable. To prevent serious distortion of the lung structure caused by tissue shrinkage, we applied tissue fixation with PFA before starting the organic-based solvent tissue clearing. Next, the tissues were sequentially immersed in DBE and ECI for complementary RI matching, which yielded transparency of the lung that was superior to that with either solvent alone; this is normally done in other tissue-clearing protocols (13–15). These advanced tissue processing and optical clearing steps enabled 3D multiplexed fluorescence microscopy for the interrogation of immune infiltrates in the asthmatic mouse lung at multiple scales.

Using the optimized protocol, we were able to conduct quantitative 3D spatial analysis of immune cell infiltrates in the mouse asthmatic lung at the organ, tissue, and cellular levels. LSFM revealed that the asthmatic lung was occupied by a volume of CD45⁺ hematopoietic immune cells that was more than 25 times larger than seen in the normal lung. Heterogeneous distribution of hematopoietic immune cells in segmented lobes and trachea was also observed. Although inconsistent delivery of allergens via intranasal instillation cannot be excluded, the uneven distribution might reflect the irregular formation of mucosal lymphoid tissues adjacent to airways. On the contrary, most CD45⁺ hematopoietic immune cells were localized close to the surface of the airways ($\leq 1,000 \mu\text{m}$) as seen in 3D LSFM images. However, some of these cells were observed inside the delineated airway segments, in

agreement with our prior observations of immune infiltrates in the inflamed airway lumen through the smooth muscle barrier (19, 20). In the tissue-level spatial assay, recognition of αSMA expression patterns enabled the segmentation of the PAs and AWs in 3D CLSM images of the asthmatic lung macrosection. In the 3D CLSM images, a larger accumulation of CD45⁺ hematopoietic immune cells was found around the PAs rather than the AWs. Postcapillary venules are known to be the main route of immune cell trafficking in asthma disease (35). However, other reports also showed that an influx of CD4⁺ and CD8⁺ lymphocytes infiltrated via the perivascular capillaries, which surround the PAs in murine lungs after asthma induction (36–39). Therefore, a possible explanation of this observation is that accumulated CD45⁺ cells in the periarterial regions (Figures 3D and 3E) infiltrated via the perivascular capillaries and moved toward the inflamed sites. Distinct immune cell populations were detected in normal and asthmatic lungs at cell resolution by 3D CLSM imaging. In the IF images of CD45 and CD3, many immune cells in the asthmatic lung strongly expressed both CD45 and CD3, whereas most immune cells in the normal lung expressed either CD45 or CD3. However, further cell marker identification is needed for accurate immune cell phenotyping.

Similar results might be obtained independently by LSFM and CLSM in different mouse lungs with the same asthma induction, but we often observed significant differences in inflammation patterns among tissues or even regions within the same lung by microscopy imaging. For example, we observed distinct distributions of CD45⁺ hematopoietic immune cells in two mouse asthmatic lungs. In Figure E7, the left image (pattern A) is the same asthmatic lung as in Figure 1, and the right image (pattern B) is another asthmatic lung. Both asthmatic lungs were prepared by the same asthma induction method in two randomly selected mice

described in METHODS. This phenomenon likely results from mouse-to-mouse variation or unintended changes in the preparation of the mouse model. Accordingly, our sequential LSFM–CLSM imaging method could be used as a tool for the quality control of lung tissue to provide consistent and reproducible results for preclinical research in asthma and other pulmonary inflammatory diseases. Application of this workflow would also prevent any biased interpretation of image data from separate LSFM and CLSM assays in multiple lung tissue samples. Furthermore, in the study of early-stage and localized lung infection in a mouse model, direct tissue sectioning and high-resolution microscopy would not be effective to identify and visualize a small and random pathological region in the lung tissue. However, by adapting our method, 3D LSFM imaging of the whole lung tissue can help localize regions of interest and thereby guide tissue sectioning and CLSM imaging to obtain cellular and molecular information.

Although demonstrated here as a tool to examine immune cell infiltration, our method could be adapted to evaluate the distribution and antiinflammatory effects in mouse lungs of drug candidates for asthma therapy. Furthermore, integration with a multiplex fluorescence assay method, such as cyclic IF imaging (40), would enable *in situ* determination of immune cell subsets and their spatial locations and interactions in the lungs. Therefore, we anticipate that multiresolution 3D fluorescence microscopy can be used broadly as a new imaging assay tool to facilitate novel discoveries in preclinical studies of asthma and other inflammatory lung diseases. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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