Point-by-Point-Reply

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We confirm that we carefully proofread our manuscript.

2. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

The revised version of our protocol exclusively contains sentences written in the imperative tense. We avoided the use of terms like "could be", "should be" and "would be". Some few more notes were included into the protocol.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: eFluor, Imaris, Bitplane, etc.

The following terms have either been deleted or replaced by generic terms: MicroBeads, human (Miltenyi Biotec), eFluor 670, LaVision UltraMicroscope II Light Sheet Microscope, ImspectorPro software, Imaris (Bitplane), Fiji.

4. 2.1.1-2.1.3: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."

We optimized the text of the indicated paragraphs as recommended.

5. 4.5: Please specify the rotation speed.

We use a rotation speed of 31 rpm. This information has now been included into the protocol.

6. 5.2 and sub-steps: Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps. For instance, what zoom factor is considered to be appropriate (5.1.6)? What sheet width is selected (5.1.7)? What are the start and end positions of the z-stack (5.1.10)?

More specific details including step-by-step bottom clicks in the software were now added to the section “5.2 Adjust settings at the light-sheet microscope to detect human cells in the context of the whole organ”. Additionally, numerical values for low and high magnification (6.3x and 32x, respectively), sheet NA (20 – 40 %) and the number of z-stack (300 - 800) are now indicated. However, ranges rather than exact values were chosen, since exact values critically depend on the specific tissue size and its positioning within the sample chamber and have to be determined for a specific experiment individually.

7. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

As suggested by the editors, we combined several of the shorter Protocol steps.

8. Please include single-line spaces between all paragraphs, headings, steps, etc.

The format was adopted accordingly.

9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted selected steps that should be visualized (about 2.5 pages).

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

All aspects have been considered.

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We confirm that all relevant details have also been highlighted.

12. Figure 2: The labels on the scale bars are hard to read; please either enlarge or define the scale bars in the figure legend.

As suggested, we replaced and enlarged the scale bars.

13. References: Please do not abbreviate journal titles.

We now included full journal titles into References.

Comments provided by Reviewer #1:

Manuscript Summary:

Schulz-Kuhnt et al. describes a method of using light sheet microscopy to image lungs of immunodeficient animals with airway inflammation and added purified human T cells. This follows a model published by these authors describing a similar method imaging T cells in the gut.

Major Concerns:

While the introduction focuses on homing of T cells, it is not clear how this method can shed additional light on T cell homing to specific areas of the lung, especially as homing is dynamic and this study uses fixed tissue.

We thank the reviewer for thoroughly assessing our manuscript and providing helpful comments. The here described transfer of human T cells into recipient mice in combination with subsequently performed light-sheet fluorescence microscopy can indeed serve as a valuable tool to monitor lung homing of primary human immune cells, as the systemic circulation and organ distribution of transferred cells occurs within the living animal **prior to tissue fixation**. As described within the first NOTE of step 4 of our protocol, recipient mice were sacrificed only 3 hours after the transfer of human T cells, indicating that there is a period of 3 hours, in which *in vivo* lung homing is supposed to take place. Fixation of lung tissue is performed only in already sacrificed animals (see step 4.3 of our protocol) and allows to preserve the before *in vivo* achieved status of pulmonary immune cell infiltration.

Many studies have using live imaging of lung intravitally to image immune cells in lung (see Lefrancais Nature 2017; Headley Nature 2016; Looney and Bhattacharya ARP 2014). These methods have also been used to image inflamed lung during airway inflammation (Hammad et al. Nat Med 2009; Bose et al. Plos One 2015), just to name a few. Additionally, new methods to image lung with light sheet microscopy have been published (Erturk et al. JOVE 2014; Mzinza et al. Cellular and Molecular Biology 2018). Note the publication in JOVE 2014. There is no reference to these other studies and how this method complements other methods of imaging lung.

The Introduction and Discussion of our manuscript have now been revised taking into account this comment. Indeed, the suggested discussion of the above mentioned publications relevantly improved the embedding of our manuscript into the current scientific context.

Besides light-sheet fluorescence microscopy, 2-Photon microscopy represents another very powerful tool for advanced lung imaging, which allows a detailed visualization of immune cells within the pulmonary microcirculation, but is clearly limited by a confined z-axis penetration. In contrast, the here applied light-sheet fluorescence microscopy of chemically cleared tissue shows a deep tissue penetration and thereby allows to quantify and describe

the intrapulmonary distribution of infiltrating immune cells within a complete pulmonary lobe. Accordingly, we now discuss that sequential performance of 2-photon microscopy and light-sheet fluorescence microscopy might represent a beneficial strategy to obtain, in the same experiment, a quantitative overview of the distribution and localization of human immune cells within the murine lung (light-sheet fluorescence microscopy) and, in addition, gain more detailed insights into cellular or microvascular processes (2-photon microscopy).

Compared to other studies, which already introduced light-sheet fluorescence microscopy as an advanced imaging tool in the field of pulmonology, our protocol shows the unique feature that it allows to characterize the lung accumulation and intrapulmonary distribution of primary human immune cells under experimentally defined inflammatory conditions. Thus, it combines the advantages of light-sheet fluorescence microscopy with the chance to directly analyze patient-derived immune cells in an *in vivo* scenario and thereby to potentially identify functional alterations imprinted by a particular disease or therapeutic regimens.

Light sheet microscopy depends on tissue clearing, and there are many methods that have different effects on tissue architecture. 4.7 does not specify what type of tissue clearing was used. There should be discussion of how tissues were cleared and how clear the tissue looked. An image of cleared lung would be helpful.

We have now specified the type of applied tissue clearing ("solvent-based clearing of the tissue using ethyl cinnamate") within the protocol and included a photo of successfully cleared lung tissue into Figure 1. Moreover, some important advantages of ethyl cinnamate-based protocols for tissue clearing have been described in the Discussion of the revised manuscript.

In the microscopy section it would be useful to be more quantitative. For example, 5.1.6 states "use low magnification", meaning 4X? 10X? More specificity would be helpful.

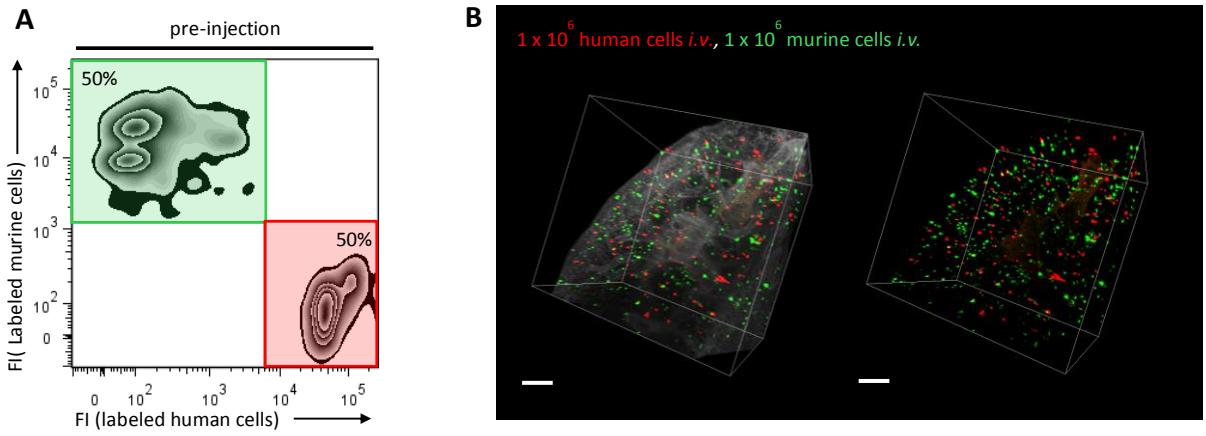
We thank the Reviewer for indicating this valid point. As suggested, we included more details in the microscopy section (see 5.2) including applied zoom factors (6.3x for low magnification and 32x for high magnification), sheet NA (20 - 40 %) and the number of z-stacks (300 - 800) that were usually acquired. We hope that these new details will improve reproducibility of our protocol.

Human T cells tend to be larger than mouse T cells, could this impact retention of T cells in small capillaries in lung? There is no quantitation or imaging of small vessels or which T cells remain in capillaries versus have extravasated. There should be some demonstration that these two populations can be distinguished.

It still remains challenging to image small pulmonary capillaries reliably via light-sheet fluorescence microscopy, even upon optimal clearing conditions. However, there exist alternative experimental strategies, which can be applied to confirm, characterize and/ or quantify extravasation of transferred human immune cells. These strategies (*e.g.* sequential

performance of 2-photon microscopy and light-sheet fluorescence microscopy) have now been discussed more extensively in the revised version of the manuscript.

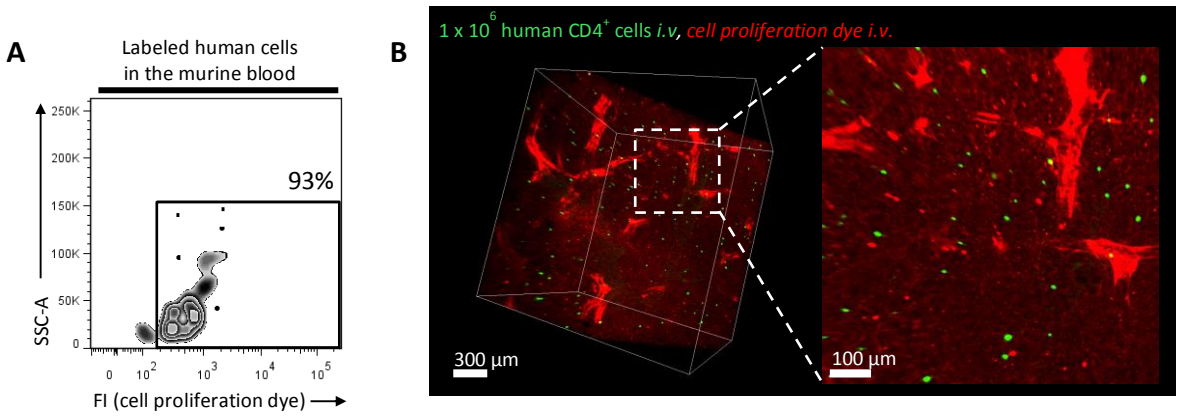
In order to experimentally address the Reviewers' concern that the larger size of human T cells and their potential mechanical retention within small lung capillaries might underlie the described accumulation of transferred human cells within the murine lung, we now performed a competitive transfer of human and murine immune cells into the same recipient animal. The acquired results are depicted below (**Point-by-Point-Reply Figure 1**) and clearly indicate that the intrapulmonary distribution pattern did not differ between transferred murine and human immune cells. For sure, the number of lung accumulated human cells was not higher than the number of murine cells, arguing against mechanical retention of human cells within murine lung capillaries.



Point-by-Point-Reply Figure 1: Competitive lung homing of human and murine immune cells. Mice with papain-induced airway inflammation were injected *i.v.* with a 50:50 mix of differently labeled human and murine cells. Human lymphoid cells derived from peripheral blood were stained with a cell proliferation dye with a peak emission of 670 nm. Murine spleen cells were stained with a cell proliferation dye with a peak emission of 580 nm. After 3 h, lung tissue was harvested and further prepared for light-sheet fluorescence microscopy. **(A)** Representative flow cytometric plot confirming equal amounts of differently labeled human (red) and murine lymphoid cells (green) within the prepared cell suspension prior to injection. **(B)** Representative 3D reconstructions of murine lungs imaged via light-sheet fluorescence microscopy. Scale bars represent 300 µm. Results are representative for n = 2. FI, fluorescence intensity.

In the Discussion of the revised manuscript, we now mention an interesting flow cytometric strategy for quantifying the extravasation of adoptively transferred immune cells within lung tissue (Galkina *et al.*; 2005). In this study, a single intravenous injection of a fluorescence-conjugated anti-CD8 antibody shortly before resection of the lung resulted in an exclusive and complete labeling of before transferred $CD8^+$ T cells within the lung vascular compartment, while already extravasated $CD8^+$ T cells within the lung interstitium remained unstained. Adapted to this experimental concept, we now performed a first pilot

experiment, in which we performed a single intravenous injection of a fluorescent cell proliferation dye shortly before resection of the lung. The used fluorophore differed from the one used for *ex vivo* labeling of human cells prior to transfer. Thus, intravascular human cells (double-positive for both fluorophores) could potentially be distinguished from extravasated human cells (single positive for the fluorophore, which was used for *ex vivo* labeling of human cells prior to transfer). As depicted in **Point-by-Point-Reply Figure 2A**, the vast majority (> 90 %) of human cells within the blood of recipient mice could indeed be characterized as double positive, thus confirming a successful labeling with the intravenously injected proliferation dye. Interestingly, subsequently performed light-sheet fluorescence microscopy of perfused and cleared lung tissue demonstrated that all retrieved human immune cells turned out to be negative for the intravenously injected proliferation dye (**Point-by-Point-Reply Figure 2B**), implicating that these cells had successfully left the pulmonary vasculature.



Point-by-Point-Reply Figure 2: Lung homing of *i.v.* injected human CD4$^+$ cells in the described *in vivo* airway inflammation model. 1 x 10^6 labeled human CD4$^+$ cells were injected *i.v.* into C57BL/6J mice with papain-induced airway inflammation. 3 h after the adoptive cell transfer, a cell proliferation dye with a different peak emission was injected *i.v.* to label intravascular cells. Afterwards, mice were sacrificed and murine blood and lung tissue were analyzed via flow cytometry and light-sheet fluorescence microscopy, respectively. **(A)** Representative flow cytometric plot gated on labeled human CD4$^+$ cells in the murine blood. **(B)** Representative 3D reconstruction of murine lung tissue showing extravasated human CD4$^+$ cells in green and intravascular cells stained with the *i.v.* injected dye in red. FI, fluorescence intensity.

Comments provided by Reviewer #2:

Manuscript Summary:

The manuscript described a technique to image lung infiltration of T cells. The similar lung fixation has been reported before. The light-sheet fluorescence microscopy looks like new to image the lung, but this microscopy has been described.

We would like to thank the reviewer for providing us with helpful comments. In the revised version of the manuscript, it has now been clearly stated that light-sheet fluorescence microscopy has already been established successfully as an important tool for advanced lung imaging. Moreover, we now refer to already published protocols describing *in situ* lung fixation.

Major Concerns:

The title of manuscript is confusing and not correct. In the study, the authors used C57 mice rather humanized mice. Secondly, there is not quantitative analysis of images, so it is confusing to use "Quantitative imaging" term.

We have changed the title of our manuscript, taking into account the Reviewers' concerns.

Comments provided by Reviewer #3:

Manuscript Summary:

This is a very interesting study where the authors were able to demonstrate in vivo monitoring of human lymphocytes infiltration into the lungs with induced inflammation in a mouse model.

We thank the Reviewer for this positive feedback.

Major Concerns:

none

Minor Concerns:

1. minor editing of the manuscript is required.

We now prepared a carefully revised version of our manuscript, which includes a slightly extended Discussion, additional subfigures and some clarifications regarding the described experimental procedure. Overall, we hope that we were able to further improve the quality of our manuscript.

2. not sure why some parts of the manuscript was highlighted with yellow color. Please remove the highlight during revision.

Based on the JoVE guidelines for manuscript preparation, we highlighted parts of the Protocol in order to indicate the essential steps of the protocol, which should later be visualized in the video. As this highlight is necessary for the further editorial processing of our manuscript, we did not remove it.