ARTICLE

**Phenotypical**

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**Keywords**

Innate lymphoid cells, IL-33, systemic inflammation model

**Abbreviations**

FOV Field of view, tissue region

ILC Innate lymphoid cell

MELC Multiepitope ligand cartography

**Abstract**

**INTRODUCTION**

**T**he

ILCs and ILC subtypes

Lung gut axis

Spatial analysis

Pascual-Reguant et al described human ILCs to localize in fibrovascular niches in inflamed tissues such as the tonsil (REF).

* Adventitial cuffs (Molofsky)

**RESULTS**

**Combining cyclic IF with a systemic inflammation model to study ILCs niches in murine lung and SI tissues**

As innate immune cells, ILCs are potent sensors of changes in their microenvironment and are able to shape their surrounding by the potent secretion of cytokines. This makes a spatial characterization of ILCs in the context of inflammation desirable. For this, we decided to use the multiplex histology technique multi epitope ligand cartography (MELC).

First, we designed an antibody panel for MELC with the aim of identifying ILCs, their subtypes, as well as other immune and non-immune cell types that would enable subsequent spatial neighborhood analysis of ILCs (Fig. 1A). As general inclusion markers for ILCs, we established CD127 and CD90.2. The ILC signature transcription factors TBET, GATA3, and RORγt, as well as the NK cell transcription factor EOMES were included to differentiate between ILC1s, ILC2s, ILC3s, and NK cells, respectively. Although we tested various GATA3 antibodies, the resulting staining was either weak or unreliable. As ILC2s are described as the predominant ILC subtype in murine lung, and with this, it is important to reliably include GATA3 in the panel, we decided to use a GATA3eGFP reporter mouse strain. Combining the GATA3eGFP reporter with an anti-GFP antibody resulted in reliable staining of GATA3 in murine lung and intestinal tissues. Additionally, we established other markers that have been previously described in the context of ILC biology, such as KLRG1, ICOS, NKp46, CCR6, and AREG. CD3, B220, Kappa/IRF4, and CD68/CD11c were included in the panel to identify T cells, B cells, plasma cells, and myeloid cells, and to distinguish them from ILCs. To differentiate T helper from T cytotoxic cells, CD4 and CD8a was used. The proliferation marker Ki67 was included as well as MHCII as a marker for antigen-presentation. We also established various structural markers including epithelial and stromal markers, such as EMCN, CD31, LYVE1, and EpCAM. Nuclei markers were included in the panel, so that DAPI was stained in the first cycle of MELC, while Sytox Green was stained in the last round of acquisition, and hence, could be used to evaluate tissue quality and integrity of each experiment.

As ILCs only constitute around 0.15 % of the immune cell compartment in murine lung (Sadeghalvad et al. 2023) and as we were not only interested in the spatial distribution of ILCs under healthy conditions, but also if and how a potential ILC niche is influenced by an inflammatory stimuli, we decided to combine MELC with a systemic inflammation model based on consecutive IL-33 i.p. injections that has been described to trigger a strong type 2 response in various organs and activate ST2+ ILC2s (REF). In preliminary experiments, we acquired a small MELC dataset of murine lung samples 3, 6, and 28 days after IL-33 application and saw a high increase in ILC2s at day 3 of IL-33 application (data not shown). Therefore, and as ILCs are innate immune cells, that are tissue-resident and potent sensors of their surrounding, we decided to focus on very early time points of inflammation, namely day 1, 2, and 3 after the onset of inflammation using IL-33. 12- to 14-week-old females of GATA3eGFP reporter mice were i.p.-injected with 300 ng IL-33 on up to three consecutive days, organs were isolated 24 hours after the last dose of IL-33 and prepared for MELC (Fig. 1B).

Taken together, we established a 40-marker MELC panel in murine lung tissue and combined this with a systemic inflammation model based on consecutive IL-33 injections where we focused on very early time points after the onset of inflammation. Using the microscopy-based method MELC.

**Major immune and non-immune cells are annotated from cyclic IF data using the Seurat workflow**

We acquired MELC data of murine lung samples 1, 2, and 3 days after IL-33 application as well as healthy controls. Analysis was performed as described in the methods part. Dimensionality reduction and cluster analysis of the lung data resulted in three clusters for the first level of annotation (AL1) and were annotated based on the respective feature profiles (Fig. 2A). Thereby, immune cells showed high levels of the pan-leukocyte marker CD45, as well as different markers for T cells, B cells, Plasma cells, and myeloid cells, such as CD3, B220, Kappa, CD68. The cluster annotated as stromal cells was high in endothelial and fibroblast-associated markers EMCN, CD31, LYVE1, PDPN, Sca1, and PDGFRa, while the cluster annotated as epithelia was marked by a high level of EpCAM and CD138.

For visual validation, the annotated cell types, namely immune cells (Darkcyan), stromal cells (Darkmagenta), and epithelia (Gold), of each FOV were depicted in x and y (Fig. 2 B) and were compared to the respective MELC IF overlays of CD45 (Cyan), CD31 (Magenta), and EpCAM (Yellow) showing correlating distribution patterns (Fig. 2C).

**Three ILC subtypes are resolved and ILC2s represent the predominant subtype in murine lung tissue**

To increase the granularity, we re-analyzed the clusters by only including markers that were associated with the respective cell type of interest in the dimensionality reduction and cluster analysis. For example, for the re-analysis of the immune compartment, only immune markers from the MELC panel were included. This approach enabled us to separate a CD3- CD127+ CD90.2+ cluster of ILCs that additionally had high levels of GATA3eGFP and KLRG1, from other immune cell types like T helper cells, T cytotoxic cells, myeloid cells, and B cells & plasma cells in AL2 (Fig. 3A). Furthermore, we were able to identify three ILC subtypes and annotate the clusters based on their feature profiles as NK cells/ILC1s, ILC2s, and ILC3s (Fig. 3B). Additionally, visual validation was performed (Fig. 3C-E). All three clusters showed high levels of GATA3eGFP (Fig. 3B). The cluster annotated as ILC2s had high levels of both CD127 and CD90.2, as well as the highest level of GATA3eGFP and additionally high levels of KLRG1 (Fig. 3B, Fig. 3C). NK cells/ILC1s only partly showed CD127 and CD90.2 but were marked by high levels of EOMES and TBET (Fig. 3B, 3D). The cluster annotated as ILC3s had levels of CD127, CD90.2, RORγt and partly TBET.

We also performed re-clustering of the stromal compartment which resulted in three subtypes: EMCN+ CD31+ blood vessels, LYVE1+ CD90.2+ lymphatics, and a CD45- IRF4+ cluster (data not shown). In summary, we were able to identify 11 different immune and non-immune cell types in murine lung MELC data, including the ILC subtypes NK cells/ILC1s, ILC2s, and ILC3s (Supplementary Fig. 1).

**Lung ILC2s are the predominant ILC subtype in murine lung and only increase at IL-33 day 3**

We first quantified the total count, as well as the proportions of the identified ILC subtypes within the immune and the ILC compartment under healthy conditions (Fig. 4A-C). The median number of identified ILC2s per FOV was 11, while the median number of NK cells/ILC1s was 8 (Fig. 4A). ILC3s were the rarest ILC subtype and were only detected in one FOV under healthy conditions (Fig. 4A). Both within the immune compartment and the ILC compartment, ILC2s represented the predominant ILC subtype, and the frequency of ILC3s was less than 1 % (Fig. 4B-C).

Next, we quantified the cellular distribution at different time points of IL-33 application (Fig. 4D-I). Comparing the time points after IL-33 application to the controls revealed a small significant increase in the total count of the analyzed cells per FOV in general at IL-33 day 1 (Fig. 4D), as well as the immune cells (Fig. 4E), and ILCs (Fig. 4F). However, a strong significant increase compared to the healthy tissue was observed after three doses of IL-33 day.

Looking at the ILC compartment, the total counts of NK cells/ILC1s (Fig. 4G), ILC2s (Fig. 4H), and ILC3s (Fig. 4I) significantly increased at IL-33 day 3 compared to the healthy control. There was no significant change in the frequencies within the ILC compartment, except for the ILC3 frequency significantly increasing by 8 percent points to a median of 8 % at IL-33 day 2 compared to healthy controls where the median was 0 % (data not shown).

The data confirmed that ILC2s represented the predominant ILC subtype in murine lung tissue. The application of IL-33 significantly triggered the accumulation of cells in general, immune cells and ILC subtypes in the lung at IL-33 day 3.

**Spatial analysis reveals accumulation of ILC2s in peri-lymphatic niches shared with myeloid cells in the lung**

Next, we wanted to analyze spatial alterations of the different ILC subtypes at different points after IL-33 application and see whether the inflammatory stimuli affected the distributions of ILC subtypes within the tissue. For this, we performed co-enrichment analysis of the identified ILC subtypes using the R packages Giotto (REF) and VoltRon (REF). Additionally, we used another R package SPIAT for measuring the minimal distance of a cell type to a reference cell and for calculating cells in neighborhood (CIN) values representing the proportions of cell types in a defined radius around the reference cell.

Spatial analysis with revealed high co-enrichment scores of ILC2s and lymphatics (Fig. 5A). This was confirmed by visual inspection and localization of ILC2s close to CD90.2+ LYVE1+ endothelial structures that represented lymphatics in IF overlays (Fig. 5B). ILC2s possessed with a median of 33 µm the smallest minimum distance to lymphatics within all measured immune cells under healthy conditions in murine lung (Fig. 5C). At IL-33 day 3, an overall decrease of the minimal distance of all tested different immune cells to lymphatics was observed, again, ILC2s had with 26 µm the smallest minimum distance (Fig. 5D). The CIN analysis revealed that within a 15 µm radius around ILC2s, lymphatics represented 4 % under healthy conditions and 6 % at IL-33 day 3 (Fig. 5E).

Besides the co-enriched localization of ILC2s with lymphatics, spatial analysis also showed spatial association of ILC2s with myeloid cells (Suppl. Fig. 2A) and ILC2s (Suppl. Fig. 2B). This went in line with high proportions of both myeloid cells and ILC2s observed in the CIN analysis of ILC2s (Supp. Fig. 2C). We did not find a global pattern of co-enrichment of ILC2s with other immune and non-immune cell types like T helper cells (Suppl. Fig. 2D).

Taken together, our data supports the accumulation of ILC2s in a peri-lymphatic niche shared with myeloid cells.

**Lung NK cells/ILC1s accumulate in peri-vascular tissue areas**

Next, we analyzed the spatial distribution of the identified NK cells/ILC1 cluster. Unlike ILC2s that showed to be spatially enriched close to lymphatic vessels, NK cells/ILC1s were co-enriched with EMCN+ CD31+ endothelial blood vessels (Fig. 6A). Of note, blood vessels were the most abundant cell type identified, while NK cells/ILC1s were rare. However, the minimal distance of NK cells/ILC1s to blood vessels was only 10 µm under healthy conditions which was significantly less compared to ILC2s and myeloid cells (Fig. 6B). This distance even decreased to only 7 µm at IL-33 day 3, which represented a significant difference compared to all other immune cell types (Fig. 6C). Additionally, the distribution of NK cells/ILC1s away from lymphatics, in peripheral tissue areas marked by endothelial blood markers was confirmed visually by IF overlays (Fig. 6D). We also investigated a potential localization pattern of ILC3s, but did not find a predominant pattern of spatial co-enrichment of ILC3s with another cell type (data not shown).

Our data suggests a difference in the spatial niche of NK cells/ILC1s localizing in peri-vascular tissue areas of the murine lung compared to ILC2s sharing their peri-lymphatic niche with myeloid cells.

**ILC2s and a mixed cluster of NK cells/ILC1s/ILC3s are resolved in murine SI tissue**

We used the same approach for identifying and annotating cell types as in the lung dataset. In AL1, we separated major cell types, namely immune cells, stromal cells and epithelia (Suppl. Fig. 3A) and performed visual validation using IF overlays (Suppl. Fig. 3B). Re-analysis of the different identified clusters separately enabled us to resolve two epithelial clusters, namely epithelia I and epithelia II; 3 stromal/endothelial clusters, namely lymphatics, blood vessels and fibroblasts; and the immune cell types, myeloid cells, B cells and plasma cells (Fig. 7A, Suppl. Fig. 4A-B).

The identification of different immune and non-immune cell types was successful except for the differentiation of T cells and ILCs. For this, we combined the cells from the mixed ILC/T cell clusters and applied a threshold on the intensity level of CD3 (Fig. 7B). Re-clustering of the CD3+ and CD3- population separately resulted in ILC/NK cell and T cell clusters annotated based on the respective feature profile (Fig. 7C). T cytotox. cells showed CD3 as well as high levels of CD8a, while T helper cells were also CD3+ but with high levels of CD4. One CD3- cluster was marked by high levels of EOMES, and partly had CD127, CD90, CD4, and RORγt, while being negative for CD3, and was annotated as ILC1s/ILC3s/NK cells. With GATA3eGFP, CD127, CD90.2 in the absence of CD3, the other ILC cluster possessed an ILC2 phenotype. We also identified one cluster that showed no levels of CD3 but high levels of CD8a, and partly CD90.2. Visual inspection revealed an intra-epithelial localization of those cells and the round shape of lymphocytes (Suppl. Fig. 6). Therefore, we annotated them as CD8+ CD3- intra-epithelial lymphocytes (IEL).

**Intestinal ILC2s increase after one dose of IL-33**

In the SI, the acquired tissue regions showed two predominant micro-anatomical structures, namely intestinal villi and intestinal lymphoid follicles (ILF). Based on this, we grouped the acquired FOVs in SI villi (Suppl. Fig. 7A) and SI ILF (Suppl. Fig. 7B) and quantified the data separately. First, we quantified the proportion of ILC subtypes.

71 NK cells/ILC1s/ILC3s and 46 ILC2s in SI villi

148 NK cells/ILC1s/ILC3s and 161 ILC2s in SI ILF

* Quantification of ILCs in Si villi regions at different IL-33 time points
* Spatial analysis of ILC subtypes in Si villi regions
* Co-enrichment of cells of the cluster NK cells/ILC1s/ILC3s with cells of the epithelia II cluster under healthy conditions, but not after IL-33 application
* Shift of distribution pattern from basal during inflammation

**DISCUSSION:**

**Establishing MELC and IL-33 systemic inflammation model**

, a field of view with a size of 665 x 665 µm normally showed around

850 total cells and approximately 250 immune cells meaning that only 2 to 3 ILCs per captured

area were expected (assuming homogeneous distribution). ILC2s

, and with this, only

around one ILC2 would be expected to be detected in all three acquired areas of each mouse –

again, simplifying and assuming homogeneous distribution of ILC2s.

**Data analysis, cell type annotation and validation**

Epithelial cluster expresses EpCAM and CD138. CD138 in murine lung can be expressed by epithelial cells

**Quantification and spatial analysis of ILCs in lung**

**Quantification and spatial analysis of ILCs in SI**

**Data Limitations and Perspectives (maximum 250 words)**

* Segmentation
* Analysis time

**MATERIAL AND METHODS**

**Mice**

**Ethical statement**

The research presented in this manuscript complies with all relevant ethical regulations. All experimental procedures involving animals were carried out after approval of the study protocols by the Landesamt für Gesundheit und Soziales Berlin, animal license number (G). Mice used for this study were kept in the animal facilities of the DRFZ under specific pathogen free conditions, which included the maintenance of a 12-h light/dark cycle with the ambient temperature set to 22 ± 2 °C and air humidity 55 ± 10% rH. Food and autoclaved water were provided *ad libitum.*  The housing of the animals were cages that were individually ventilated (IVCs) and contained an enriched environment. Animal experiments were conducted following the 3 R Principles: replace, reduce, refine. Mice were handled using tunnels to reduce stress and anxiety.

**IL-33 application**

Female 12- to 14-week-old GATA3-enhanced green fluorescent protein (eGFP) reporter mice (van Doorninck et al. 1999) were i.p.-injected with 300 ng recombinant carrier-free mouse IL-33 (Biolegend, San Diego, USA) solved in 0.1 to 0.5 ml sterile saline on up to three consecutive days. The mice were inspected and weighted on a daily basis.

**Organ isolation and tissue preparation**

Mice were sacrificed after none, 1, 2, or 3 doses of IL-33, 24 hours after the last dose. Approximately 1.5 hours before killing the mice, *intra-venous (i.v.)* administration of 200 mg/kg bodyweight Pimonidazole (Hypoxyprobe, Burlington, USA) was performed. Mice were anesthetized and perfused with 1 % freshly prepared electron-microscopy grade paraformaldehyde (PFA) (EMS, Hatfield, USA) solution. The SI was isolated and flushed extensively with 50 ml Phosphate Buffered saline (PBS) using a syringe before incubation in freshly prepared 1 % PFA in PBS solution for three hours at 4 °C. The lungs were isolated still connected by the trachea, and incubated for three hours in 1 % PFA solution at 4 °C. The PFA solution was discarded, and the samples rinsed with cold PBS for 2-5 min on ice. Afterwards, the organs were incubated in 15 % sucrose solution at 4 °C. After 6 to 12 hours, the sucrose solution was discarded and the samples were put into 30 % sucrose solution for 6 to 12 hours at 4 °C. Lung samples were prepared by inflating the lungs through the trachea with 1:2 PBS:Tissue-Tek O.C.T. Compound (Sakura) solution using a syringe and carefully put into prefilled cryomolds containing O.C.T. medium. To prepare the SI for freezing, the SI was cut into three equal parts. Each part was then slid on blunt scissors and carefully cut longitudinally. Swiss roll samples were prepared (Moolenbeek & Ruitenberg 1981) and carefully put into cryomolds filled with O.C.T. medium.

**Cyclic IF: Multi epitope ligand cartography (MELC)**

**Tissue Preparation**

Fresh frozen tissue was cut 5 µm thick with a NX80 cryotome (ThermoFisher, Waltham, Massachusetts, USA) on 3-aminopropyltriethoxysilane (APES)-coated cover slides (24 × 60 mm; Menzel-Gläser, Braunschweig, Germany). Samples were fixed for 10 min at room temperature using a freshly opened EM grade PFA ampulla (methanol- and RNAse-free; Electron Microscopy Sciences, Hatfield, Philadelphia, USA) diluted to 2%. After washing three times with PBS, samples were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. Subsequently, a blocking step with 10% goat serum and 1% BSA in PBS was performed for at least 20 min. Afterwards, a fluid chamber holding 100 μl of PBS was created using “press-to-seal” silicone sheets (Life technologies, Carlsbad, California, USA; 1.0 mm thickness) with a circular cut-out (10 mm diameter), which was attached to the coverslip, surrounding the sample.

For every MELC run, a bottle of fresh PBS with 1% BSA and 0.02% Triton X-100 was used. The sample was placed on the sample holder and fixed with adhesive tape followed by accurate positioning of the binning lens, the light path, as well as Köhler illumination of the microscope.

**Image Acquisition**

The data was acquired using a modified Toponome Image Cycler Mm3 (Tic) (Meltec GmbH & Co. KG, Magdeburg, Germany) and a BioDecipher Device 1.0 (BioDecipher GmbH, Magdeburg, Germany). Details of equipped components are summarized in supplementary table 1.

MELC image acquisition consists of 4 steps that are repeated in cycles: (1) Antibody incubation for 15 to 55 mins and 30 automatic washing steps; (2) Image acquisition of up to 4 channels of 3 previously selected FOVs; (3) Photo-bleaching of 5 to 30 mins of each FOV; (4) Image acquisition of the bleaching image for every selected FOV. During image acquisition, image stacks of 10 in both positive and negative z-dimension were acquired sized 2048 x 2048 pixels, where each pixel represented 0.325 µm.

**Antibody Panel**

All antibodies used for this study were titrated for the optimal dilution in murine lung and SI samples and are summarized in Supplementary table 2. Optimal order of the antibodies was determined by test experiments. Steric hindrance issues that might appear due to this specific labeling order have been ruled out as previously shown.

For each experiment, 60 µl of freshly prepared antibody dilution was pipetted in a 96-well plate.

**Image Pre-processing**

Image preprocessing of the Tic MELC data was performed as previously described (Pascual-Reguant et al. 2021). For the MELC data acquired with the BioDecipher device, the implemented TICobserver software (BioDecipher GmbH, Magdeburg, Germany) was used. Both approaches comprised image registration, background subtraction, and illumination correction, and achieved comparable results. Image normalization was performed in Fiji ImageJ (Schindelin et al. 2012; Schindelin et al. 2015) just as described in previous publications, and included background estimation (rolling ball algorithm), edge removal, as well as image intensity scaling (Holzwarth et al. 2018; Pascual-Reguant et al. 2021; Mothes et al. 2023). Each staining was manually examined for artefacts and excluded when major auto-fluorescent artefacts covered a dominant area of the stained tissue. If a minor artefact was located in a part of the image, where there was no tissue or only covered an area of up to 10 cells, the artefact was cut out from the image and standardization was re-applied.

**Pixel classification using Ilastik**

Pixel classification was done using Ilastik (Berg et al. 2019). The integrated random forest algorithm was trained based on the IF overlay depicting the nuclei (DAPI or Sytox) and a Z-projection of selected membrane markers (EpCAM, CD45, CD44, CD11c, CD4, LYVE-1, podoplanin (PDPN), Sca1, CD68, platelet derived growth factor receptor-α (PDGFRa), CD138, fibronectin (FN), sialic acid binding Ig-like lectin F (SiglecF), and Kappa) to classify pixels into Nuclei, Cytoplasm, and Extacellular matrix (ECM). Training was performed separately for lung and SI data. Visual inspection and minor adjustments of each FOV resulted in optimized probability maps for Nuclei, Cytoplasm and ECM that were exported.

**Segmentation and feature extraction**

Probability maps created with Ilastik together with 16-bit greyscale images were used as input for CellProfiler 4.0 (Stirling et al. 2021) for segmentation of nuclei and cells, as well as for feature extraction and data export. For each FOV of a MELC experiment, the probability maps for Nuclei, Cytoplasm and ECM created in Ilastik, as well as all single marker images standardized, and intensity adapted were used as input in CellProfiler. Before the actual segmentation, image subtraction was performed by subtracting the ECM probability map from the nuclei probability map as well as from the cytoplasm probability map using CellProfiler’s ImageMath module. Subsequently, the subtracted nuclei image was used for segmentation of nuclei as primary objects with the module IdentifyPrimaryObjects with advanced settings.

By applying an adaptive thresholding strategy and a two class Otsu as the thresholding method segmentation of nuclei was achieved. Using the identified primary objects (nuclei) as seed points and similar settings as for the primary objects, secondary objects were identified by running the IdentifySecondaryObjetcs module. The identified secondary objects represented cells. Tertiary objects, named cytoplasm were created by subtraction of nuclei (primary objects) from cells (secondary objects) implemented in the IdentifyingTertaryObjects. In case that the segmentation outcome did not pass manual inspection, an additional step using the module EditObjectsManually was conducted, where under-segmented, clothed cell clumps and over-segmented cells were manually refined.

For each identified object, median fluorescence intensity (MFI) of nuclear markers was measured in the respective primary object (nucleus), while the MFI of all other markers was measured in the respective secondary object (cell). Resulting from the applied CellProfiler pipeline, the measured single cell features of all objects together with the respective spatial information of the X- and Y-coordinates of the nuclei were exported as csv files.

Complete and detailed CellProfiler pipelines and all data tables generated are publicly available in the Zenodo open access repository https://zenodo.org/ (see “Data availability”).

**Data analysis**

**Data tidying**

Thresholding

Data imputation

**Dimensionality reduction, cluster analysis, and cell type annotation**

**Spatial neighborhood analysis**

SPIAT

Giotto & VoltRon

**DATA AVAILABILITY STATEMENT**

<https://github.com/mikrohscopist/Murine_ILC_niches_lung_SI_IL-33>

**CONFLICT OF INTEREST DISCLOSURE**

**ETHICS APPROVAL STATEMENT**

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**AUTHOR´s CONTRIBUTIONS**

A.E.H. conceptualized the study. S.K, R.G., R.U., P.M. performed experiments. S.K. analyzed the data and S.K., A.R.P., and A.E.H. interpreted the results and wrote the manuscript. S.K., A.R.P., R.A.N., A.M. and A.E.H. discussed the results. All authors reviewed the manuscript.

A.M.

R.K. and L.P. provided technical support for the MELK and BioDecipher instruments.

**REFERENCES**

Vancouver reference style

**ADDITIONAL INFORMATION**

**Tables**

**Figures with legends**

**Supporting information**