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

IL-33 is a profibrogenic cytokine associated with type 2 inflammation, lung fibrosis and ILC2s (Li et. al, doi: 10.1016/j.jaci.2014.05.011)

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 surroundings 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 (described in detail in the methods part). 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). Visual validation was performed and confirmed correct annotation (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. 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 by median, followed by NK cells/ILC1s. ILC3s were only detectable in one FOV under healthy conditions and ILC3s were present in frequencies of less than 1 % (Fig. 4B-C).

Next, we quantified the cellular distribution at different time points after 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 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 healthy controls. 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.

As expected, compared to the lung, both SI villi and SI ILF tissue regions harbored significantly higher numbers of total cells, immune cells, and ILCs in steady state, with the highest total counts in SI ILF tissue regions (Suppl. Fig. 9A-C). Of note, the frequency of ILCs within the immune compartment did not differ significantly between the different organs and tissues (Suppl. Fig. 9D). The highest counts of ILCs, and the identified subtypes were detectable in the SI ILF regions (Suppl. Fig. 9C). Zooming into the ILC subtypes identified in the SI revealed frequencies of NK cells/ILC1s/ILC3s and ILC2s between 40 and 60 % in both SI villi and SI ILF with no significant differences (Suppl. Fig. 9E-F).

Quantification of the SI villi data at the different conditions showed that the total cell number as well as the total immune cell number significantly decreased after one dose of IL-33 compared to healthy controls in SI villi regions (Fig. 8A-B) while the number of ILCs was only significantly reduced at IL-33 day 3 (Fig. 8C). However, although the number of immune cells decreased at IL-33 day 1, the total frequency of immune cells rose significantly at the same time (Fig. 8D). Looking at the ILC frequency within the immune compartment only showed an increasing trend at IL-33 day 1 (Fig. 8E). At IL-33 day 3, not only was the total ILC count significantly reduced, but also the frequency of ILCs as well as the frequencies of ILC subtypes, namely NK cells/ILC1s/ILC3s and ILC2s within the immune compartment (Fig. 8E-G).

Like lung ILC2s, spatial analysis of ILC subtypes in SI villi regions revealed accumulation of ILC2s together with myeloid cells (Fig. 8H-I). However, we did not observe a clear pattern of co-enrichment of ILC2s and lymphatics or fibroblasts, although IF overlays suggested a spatial association (data not shown). Analyzing the spatial distribution of NK cells/ILC1s/ILC3s, we did not observe co-enrichment of NK cells/ILC1s/ILC3s with any other immune cell type, as well as fibroblasts, blood vessels, lymphatics and epithelia I cells under any analyzed conditions (data not shown). However, co-enrichment analysis revealed spatial association of the NK cells/ILC1s/ILC3s and epithelia II cells under healthy conditions (Fig. 8K). Besides strong signal of EpCAM, the epithelia II cluster was also marked by high intensities of Ki67, as well as Sca-1 and CD44 (Fig. 7A) which, together with IF overlays (Suppl. Figure 8) pointed towards a cluster identity of epithelial cells of the basal area of the villi including the crypts and intestinal stem cells (ISC), and a spatial separation from the cells of the epithelia I cells. The pattern of co-enrichment of NK cells/ILC1s/ILC3s and epithelia II cells was resolved after one dose of IL-33 pointing towards a spatial re-distribution of NK cells/ILC1s/ILC3s during inflammation, which was confirmed in the IF overlays (Fig. 8K-M).

**DISCUSSION:**

**1.Introduction—mention gaps in previous research**

This study used multiplex cyclic IF to identify ILCs and ILC subtypes in murine lung and SI tissue under homeostasis and investigate their adaptations during an IL-33 triggered inflammation. While most studies using spatial multiplex approaches rather focus on either highly abundant immune cells, often T cells and B cells in the context of tumors, we were rather interested in establishing a workflow that (I) is able to spatially resolve abundant cells as well as rare cell types in the tissue and (B) enables to perform spatial neighborhood analysis to identify conserved or tissue-specific distribution patterns of cells. Thereby, we were especially interested in ILCs, rare innate immune cells that are tissue resident and quickly react to changes in their microenvironment.

**2. Summarizing key findings—let your data speak**

We established a 40+ marker panel for MELC in murine lung and SI and analyzed samples collected from GATA3eGFP mice challenged with up to three doses of IL-33 on consecutive days representing a type 2 inflammation model. Using an optimized image and data analysis workflow consisting of different open-source software, we were able to annotate different ILC subtypes in both organs as well as other immune and non-immune cell types. Quantification of ILC subtypes in the lung at early time points of IL-33 application revealed a strong significant increase in ILCs, NK cells/ILC1s and ILC2s at IL-33 day 3. Quantification of intestinal ILC2s showed a significant increase in the frequency of ILC2s already after one dose of IL-33, however, a significantly decreased frequency on ILCs, NK cells/ILC1s, and ILC2s at IL-33 day 3. Spatial co-enrichment analysis revealed a peri-lymphatic localization of lung ILC2s and a shared niche of both lung and intestinal ILC2s with ILC2s and myeloid cells. In contrast, lung NK cells/ILC1s did not localize together with lymphatics but with blood endothelial cells, in peri-vascular niches. Co-enrichment of intestinal NK cells/ILC1s/ILC3s with other immune and non-immune cells was only observable for NK cells/ILC1s/ILC3s and epithelial II cells under healthy conditions, but not after IL-33 application.

**3. Interpreting results—compare with other papers**

Our spatial analysis confirm a bronchovascular localization of ILC2s in the lung described by Dahlgren et. al, but our co-enrichment analysis and visualizations additionally show high co-enrichment scores of ILC2s and LYVE1+ CD90.2+ lymphatics pointing towards a more specific peri-lymphatic ILC2 niche. Furthermore, lung ILC2s and ILC3s but not NK cells/ILC1s show ICOS levels. ICOS regulates ILC2 homeostasis independently of T cells and B cells and is required for the proliferation and accumulation of mature ILC2s in the lungs and intestines (Paclik et. al, doi: 10.1002/eji.201545635). It has been shown that ICOS+ ILC2s are crucial for tissue protection during the early time points of bleomycin-induced lung injury, PR8 influenza infection and LPS airway challenge (Hrusch et. al, doi: 10.1038/mi.2017.42) as well as intra-nasal application of IL-33 (Paclik et. al, doi: 10.1002/eji.201545635), but this has not yet been shown for the *i.p.* IL-33 systemic inflammation model. Furthermore, ILC2s accumulate together with myeloid cells. Identified myeloid cells in our study expressed high levels CD68, CD11c and MHC II and most likely represent both alveolar macrophages and dendritic cells (Zaynagetdinov et. al, doi: 10.1165/rcmb.2012-0366MA). A shared niche of ILC2s and myeloid cells that our spatial co-enrichment analysis revealed suggests an interaction of ILC2s and myeloid cells as described by Hrusch et. al at early time points of bleomycin-induced lung inflammation where increased ICOSL in alveolar macrophages is found (Hrusch et. al, doi: 10.1038/mi.2017.42).

Tin general, the number of ILC2s highly depends on age, sex, and other environmental conditions, but in murine lungs, it is considered very low, e.g. Sadeghalvad et. al reported an ILC2s frequency of around 0.15 % of the immune cell compartment in murine lung (Sadeghalvad et al. 2023). Quantification of the ILC2s in our lung data resulted in a frequency of ILC2s of about 5 % within the immune compartment under healthy conditions. This was much higher than expected. Co-enrichment analysis and visual inspection showed that ILC2s localize in cell accumulations. This leads to an increased micro-anatomical frequency of ILC2s and explains how rare cell types can have an important impact on the tissue scale. It also highlights the importance of spatially-resolved studies of cell in the tissue context as this information gets lost during dissociative procedures.

Our findings that point towards a functional niche of ILC2s, lymphatics, and myeloid cells also aligns with studies describing an interplay of lung ILC2s and lymphatics, where lung ILC2s stimulate lymphatics (but not blood endothelial cells) to upregulate CCL21 (via LIF-LIFR) providing a crucial migratory signal for CCR7+ (the ligand of CCL21) immune cells (Gogoi et. al, https://doi.org/10.1038/s41586-024-07746-w).

Interestingly, our findings show that while lung ILC2s reside in peri-lymphatic niches, identified NK cells/ILC1s from the lung did not localize close to lymphatics but are co-enriched with endothelial blood vessels that are marked by EMCN and CD31.

Function NK cells/ILC1s vs function ILC2s

A conserved fibrovascular niche of ILCs in different chronically inflamed human tissues has been described by Pascual-Reguant et. al (Pascual-Reguant et. al, doi: https://doi.org/10.1038/s41467-021-21994-8). We acquired data of murine lung and SI from the same mice in order investigate conserved or tissue-specific spatial and phenotypical patterns of ILCs. While analysis of the cell types and ILC subtypes worked well on the lung data, in general, data analysis was more challenging in the SI tissue and required additional steps, for instance, to separate ILC subtypes and T cell subtypes. This was most likely because of the more severe tissue destruction associated with the IL-33 induced systemic inflammation.

ILC2s in the GI Dahlgren et. al

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

**4. Addressing limitations—their potential impact on the results**

**5. Implications for future research—how to explore further**

**6. Conclusion—summarize content**

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

Number of animals and area

Working with imaging-based data several challenges compared to dissociative methods such as single cell-sequencing or flow cytometry.

* Segmentation
* Spatial cross contamination
* Analysis time
* Clustering of cells 🡪 it is not possible to check every marker as in flow and set strict gates

It was not possible to establish working stainings of cytokines.

Spatial transcriptomics methods 🡪 single cell resolution and identification of rare cell types

**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 (LaGeSo), animal license number (G0122/20). 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 analysis was performed using R (R Core Team 2020) and RStudio (RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL <http://www.rstudio.com/>) version 2023.06.1. Used packages and versions are summarized in Supplementary table 3.

**Data tidying**

Feature selection

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**