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# HUMAN INNATE LYMPHOID CELL TRAFFICKING AND FUNCTION IN VIVO

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Cover illustration: The MISTRG mouse in front of an illustration of intravascular cell labelling of cells in the vasculature.

# Human innate lymphoid cell trafficking and function in vivo

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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# POPULAR SCIENCE SUMMARY OF THE THESIS

Being alive is not easy. As we go about our daily lives, we are exposed to multitudes of things from our environment. These things could be benign but could also be harmful, either way they must be dealt with in some way. Furthermore, even merely existing for some time will cause some wear and tear to our tissues and organs which need to be repaired. Fortunately, we have a sophisticated system of cells specifically for dealing with these exact problems called the immune system.

There are different cell types in the immune system, each cell type is specialized for specific roles. One of the newly discovered immune cell types is the innate lymphoid cells (ILCs). ILCs live in barrier sites, sitting in places within organs where the tissues and cells meet the outside world. Here they function like a sentry, watching for danger signs from the environment. They are one of the first responders to threats from the environment, sounding the alarm, amplifying the signal, and calling for the appropriate immune response to deal with the threat. The way ILCs are positioned in the tissue allows them to play this role extremely well, but how do they get there in the first place? And why?

Many immune cells use the circulatory system as a highway to reach various places where they are needed and are therefore abundant in the blood, ILCs however are famous for being tissue resident. This makes it difficult to study, especially in humans. In this thesis we use a humanized mouse model (mouse engrafted with a human immune system) to investigate human ILCs in the vascular and tissue compartments of organs, specifically the lung, and how their specific location relates to their function. In Paper I and Paper II we identified two specific human ILC populations in the vasculature, the $CD5^+$ ILCs and $TCF\text{-}1^+$ proliferative ILCs that are immature and has potential to differentiate to other ILC subsets. In Paper III, we compared ILCs and their close relative natural killer (NK) cells in the vascular and tissue compartment of the lung and found that their specific location or environment influence their biological functions. ILCs and NK cells in the vasculature are more immature and focused on migration, while ILCs and NK cells in the tissue adopt more functional roles, communicate with other cells and are involved in tissue maintenance.

These findings help further our understanding of human ILCs and contribute to the development of new treatments for diseases further down the line by modulating ILC migration.

# ABSTRACT

Human organs and tissues, such as the lung, are composed of various spatially distinct anatomical compartments. Each compartment represents a different tissue environment within the organ, performs specific functions and in turn promotes the development, migration, and function of specific cell types. Innate lymphoid cells (ILCs) are innate immune cells that perform important functions as one of the first responders in immune defense and as maintainers of tissue homeostasis. ILCs are often considered as the innate counterpart of T cells, but instead on specific antigens, ILCs heavily rely on environmental signals for their development, activation, and function. Therefore, investigating the migration and spatial distribution of human ILC subsets between compartments and how it relates to their function is important. In the past several years this has been studied in both mice and humans, but human studies have been more difficult due to experimental limitations. To overcome this limitation and fully investigate human ILC trafficking and function *in vivo*, we used the MISTRG humanized mouse model in combination with intravascular cell labeling to assess human ILCs in the vascular and tissue compartments. Additional methods were employed to further investigate specific questions, for example the OP9-DL1 co-culture system to assess the differentiation potential of specific ILC subsets and single-cell RNA-sequencing to investigate ILC heterogeneity between anatomical compartments.

In Paper I, we identified a specific population of human $CD5^+$ ILCs that resides in the vascular compartment of the lungs and various organs. These $CD5^+$ ILCs are comprised of mature ILC1s and an immature population that can differentiate into mature ILCs. $CD5^+$ ILCs may function as sentinels that patrol the blood vessels and are recruited into the tissue during inflammation. In Paper II, we investigated the relationship between human ILC localization and proliferation. We identified a specific proliferative ILC population expressing the transcription factor TCF-1 that resided predominantly in the spleen and the vasculature of non-lymphoid organs. In Paper III, we investigated the heterogeneity of human ILCs and NK cells in the vascular and tissue compartment of the lungs. We found that ILC subsets are heterogenous and distributed in both vascular and tissue compartments. In contrast, NK cell subsets were strictly divided between $CD56^{dim}$ subsets in the vasculature and $CD56^{bright}$ and transitional subsets in the tissue. Furthermore, the spatial distribution of ILCs and NK cell subsets was linked to biological functions, such as migration, tissue residency, and adaptation to the tissue microenvironment.

Overall, this thesis provides new insights into human ILCs, specifically how their features and functions are regulated by their localization and the tissue microenvironment. These insights further our understanding of human ILC biology, and potentially contribute to the development of new treatments for human disease by modulating ILC migration.

# LIST OF SCIENTIFIC PAPERS

I. **Arlisa Alisjahbana**, Yu Gao, Natalie Sleiers, Elza Evren, Demi Brownlie, Andreas von Kries, Carl Jorns, Nicole Marquardt, Jakob Michaëlsson and Tim Willinger. CD5 surface expression marks intravascular human innate lymphoid cells that have distinct ontogeny and migrate into the lung. *Frontiers in Immunology*. 2021; 12:752104. DOI: 10.3389/fimmu.2021.752104

II. Yu Gao*, **Arlisa Alisjahbana***, Daryl Z. H. Boey, Imran Mohammad, Natalie Sleiers, Joakim S. Dahlin and Tim Willinger. A single-cell map of vascular and tissue lymphocytes identifies proliferative TCF-1$^{+}$ human innate lymphoid cells. *Frontiers in Immunology*. 2022; 13:902881. DOI: 10.3389/fimmu.2022.902881 *Co-first authors

III. **Arlisa Alisjahbana**, Imran Mohammad, Yu Gao, Elza Evren and Tim Willinger. Single-cell RNA-sequencing of human lung innate lymphoid cells in the vascular and tissue niche reveals molecular features of tissue adaptation. *Submitted for publication***

# SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

IV. Elza Evren, Emma Ringqvist, Kumar Parijat Tripathi, Natalie Sleiers. Ines Có Rivers, **Arlisa Alisjahbana**, Yu Gao, Dhifaf Sarhan, Tor Halle, Chiara Sorini, Rico Lepzien, Nicole Marquardt, Jakob Michaëlsson, Anna Smed-Sorensen, Johan Botling, Mikael C. I. Karlsson, Eduardo J. Villablanca and Tim Willinger. Distinct developmental pathways from blood monocytes generate human lung macrophage diversity. *Immunity*. 2021; 54(2): 259-275.e7. DOI: 10.1016/j.immuni.2020.12.004

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| $^{137}Cs$ | Caesium-137 |
| ANOVA | Analysis of variance |
| AREG | Amphiregulin |
| BAL | Bronchoalveolar lavage |
| BRGS | Balb/c Rag2 γc Sirpα mouse strain |
| CCR9 | C-C chemokine receptor type 9 |
| cGy | Centigray |
| CILP | Common innate lymphoid progenitor |
| CLPs | Common lymphoid progenitors |
| COPD | Chronic obstructive pulmonary disease |
| CRTH2 | Prostaglandin D2 receptor 2 |
| CXCL13 | Chemokine (C-X-C motif) ligand 13 |
| DLL1 | Delta-like ligand 1 (Notch ligand) |
| E4BP4 | E4 promoter-binding protein 4 |
| GATA3 | GATA binding protein 3 |
| GCPR | G-protein-coupled receptors |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HIV-1 | Human immunodeficiency virus 1 |
| HLA-DR | Human leukocyte antigen – DR isotype |
| HSPCs | Hematopoietic stem and progenitor cells |
| IFNγ | Interferon gamma |
| IL | Interleukin |
| ILCP | Innate lymphoid cell precursors |
| ILCs | Innate lymphoid cells |
| Ki67 | Nuclear protein Ki67 |
| KIR | Killer cell immunoglobulin-like receptor |
| KIRs | Killer immunoglobulin-like receptors |
| KLRG1 | Killer cell lectin-like receptor subfamily G member 1 |
| LFA-1 | Lymphocyte function-associated antigen 1 |
| LTi | Lymphoid tissue inducer cells |

| | |
|---|---|
| MAdCAM-1 | Mucosal vascular addressin cell adhesion molecule 1 |
| M-CSF | Macrophage colony-stimulating factor |
| MKI67 | Marker of proliferation Ki-67 |
| NK cells | Natural killer cells |
| NKp46 | Natural killer cell p46-related protein |
| PAP | Pulmonary alveolar proteinosis |
| PBMCs | Peripheral blood mononuclear cells |
| PI | Propidium iodide |
| PLZF | Promyelocytic leukemia zinc finger protein |
| PMA | Phorbol 12-myristate 13-acetate |
| RORγt | RAR-related orphan receptor gamma |
| S1P | Sphingosine-1-phosphate |
| S1PR1 | Sphingosine-1-phosphate receptor 1 |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| SIRPα | Signal regulatory protein alpha |
| TCF-1 | T cell factor 1 |
| TCR | T-cell receptor |
| TPO | Thrombopoietin |

# 1 INTRODUCTION

## 1.1 INNATE LYMPHOID CELLS (ILCS)

Innate lymphoid cells (ILCs) represent innate counterparts to T cells[1–6] and were identified not long ago. Similar to T cells, ILCs are commonly divided into distinct groups which includes natural killer (NK) cells, ILC1s, ILC2s, ILC3s and lymphoid tissue inducer (LTi) cells[3]. NK cells match $CD8^+$ cytotoxic T cell function while ILC1s, ILC2s and ILC3s are related to $CD4^+$ T helper subsets Th1, Th2 and Th17 in their signature transcription factors, effector cytokines and the type of pathogens they respond to. ILC1s produce interferon gamma (IFNγ) and require the transcription factor T-bet. ILC2s and produce type 2 cytokines such as IL-4, IL-5 and IL-13 are need GATA3. ILC3s produce IL-17 or IL-22 and rely on RORγt. IL-7 is critical for their development and thus helper ILCs are usually defined by the cell surface expression of IL-7Rα (CD127)[7,8]. They also do not express any lineage (Lin) markers that define T lymphocytes, B lymphocytes, dendritic cells, macrophages, granulocytes, and progenitor cells. In humans, CRTH2 and CD117 are often used as surface markers for ILC2s and ILC3s, respectively[7,9–13]. However, no equivalent surface marker has been established for ILC1s which are defined as $CRTH2^-$ and $CD117^-$ [14,15].

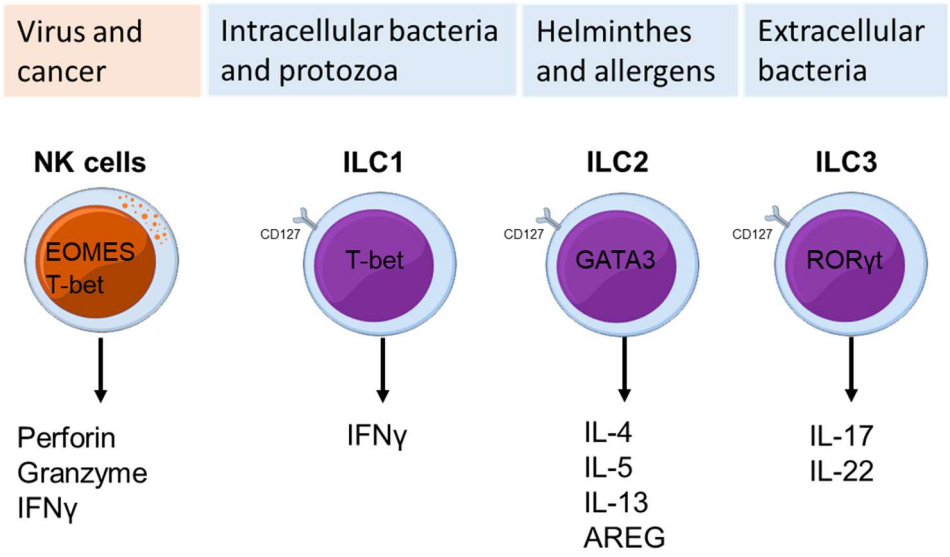


**Figure 1.** Graphical summary of the main human ILC subsets.

ILCs are enriched in mucosal tissues and, like their T cell counterparts, ILCs orchestrate appropriate immune responses against pathogens as their main immunological function. However, unlike T cells, they do not possess antigen-specific rearranged T cell receptors and do not need antigen recognition or undergo clonal expansion to function. Instead, ILCs respond directly to tissue signals or cytokines expressed by their surrounding environment[16–21]. This is in line with the finding that ILCs are mostly residing in the tissue and are rare in circulation. Their fast effector function combined with their strategic position in barrier sites makes them

important first responders of the immune system[1,22]. ILCs and T cells regulate each other and orchestrate the correct immune response to various immunological threats.

Apart from host defense, ILCs perform various other important functions to promote tissue homeostasis. For example, ILCs are involved in maintaining tissue tolerance and repair of damaged tissue. ILC2s produce amphiregulin to promote epithelial growth and repair after injury[23,24]. Furthermore, IL-4 and IL-13 produced by ILC2s promote tissue remodeling and wound healing though the differentiation of alternatively activated macrophages. In adipose tissues, ILC2s are also involved in thermogenesis[25,26]. ILC3s in particular are responsible for host-microbiota crosstalk[27,28] and a dysfunctional ILC3 response in the intestine leads to a loss of immune control over the microbiota.

A unique characteristic of ILC1s is the expression of surface markers commonly found in T cells, such as CD4, CD5, CD6, CD28 and CD27[29–32]. ILC1s are also known to express intracellular CD3ε and δ proteins[30,32,33] as well as transcripts for T cell receptor (TCR) transcripts[20,29,32]. This has generated controversy on whether ILC1s are a *bona fide* ILC subset or contaminating T cells[34–38]. Interestingly, intracellular CD3ε and δ proteins are also present in human NK cells from fetal liver[39], thymus and cord blood[40] and in human NK cells derived from fetal liver, thymus and cord blood in the presence of Notch ligands *in vitro*[40,41]. This suggests that these ILC1s are immature instead of being contaminating T cells. Two studies support this, proposing that $CD5^+$ ILC1s expressing intracellular CD3 are precursors of mature ILC subsets[33] and $KIR^+NKG2A^-$ NK cells[32].

Collectively, this shows that ILC1s are heterogenous, containing various mature and immature subsets. Further investigation on defining ILC1 identity, heterogeneity and its relation to NK cells, T cells and other ILC subsets is needed.

## 1.2 ILC DEVELOPMENT

ILCs appear early in embryonic development[42] and are important for the development of secondary lymphoid organs[43]. Shortly after birth, they are also crucial for developing tolerance to the microbiota[44]. Adult ILCs are understood to develop from hematopoietic stem and progenitor cells (HSPCs) in the bone marrow.

The ontogeny of mouse ILCs and NK cells has been investigated extensively, which has been reviewed[45,46]. These studies rely on *in vivo* techniques such as transcription factor reporter mice, which are unavailable for humans. Consequently, studies on human NK and ILC development have been mostly limited to *in-vitro* differentiation studies. All human ILC and NK cell subsets can be generated *in vitro* from human $CD34^+$ HSPCs of cord blood and bone marrow origin when cultured with OP9 cells or mesenchymal stem cells and specific cytokines[32,47]. For *in-vivo* studies of human ILCs, adoptive transfer into humanized mouse models has emerged as an important tool to confirm precursor-product relationships between subsets.

In general, human NK and ILC development follows a similar path as in mice and several human precursors have been identified. For example, the human equivalent of mouse common innate lymphoid progenitors (CILPs) giving rise to both NK cells and ILCs was described by Scoville et al. as $CD34^+$ $CD117^+$ $CD45RA^+$ $IL1R^+$ CILPs that express RORγt[48]. This subset is able to develop into NK cells and all ILC subset *in vitro*, but only generates NK cells *in vivo*. Recently, a different study by Tufa et al. describes the human CILP as $CD34^+$ $\alpha4\beta7^+$ $CD117^{+}$[49]. They discovered that this subset also expresses RORγt and further subdivided the subset by the expression of CD48. $CD34^+$ $\alpha4\beta7^+$ $CD117^+$ $CD48^-$ CILPs give rise to only NK cells *in vitro* and *in vivo* whereas $CD34^+$ $\alpha4\beta7^+$ $CD117^+$ $CD48^+$ CILPs can give rise to NK cells, ILC2s and ILC3s. Both studies reveal an important difference between humans and mice by demonstrating that RORγt expression is a more general feature of human ILCs and that RORγt promotes human NK and ILC development.

According to the linear model by Freud *et al.*, the development of human NK cells from hematopoietic stem cells occurs in a stepwise fashion[50]. In this model, Stage 1 NK cell precursors express CD34, CD45RA and CD10 and are similar to common lymphoid progenitors (CLPs). At Stage 2, the precursors lose CD10 and express CD117. Stage 3 marks the beginning of a lineage restricted NK cell progenitor with the downregulation of CD34 and the acquisition of LFA-1. At Stage 4, CD94 is acquired, and the cells become $CD56^{bright}$ NK cells. Finally, at Stage 5 cells become mature $CD56^{dim}$ NK cells by downregulating CD94 and acquiring CD16 and killer immunoglobulin-like receptors (KIRs)[51]. Also, the human NK cell progenitor defined as $CD34^+$ $CD45RA^+$ $CD10^+$ $CD7^+$ has been shown to be restricted to give rise to NK cells and not to develop into ILCs[52], which supports the idea that NK cells and ILCs develop from different lineages.

Recent studies of human ILC development have also given new insight into NK cell development. In mice, NK cells and the helper ILC subsets are two separate lineages derived from CILPs. In humans however, they seem to be closely intertwined. RORγt is expressed by human ILC subsets and $CD56^{bright}$ NK cells[48] whereas it is more restricted to ILCs in the mouse [53,54]. Human ILCPs express RORγt and are able to give rise to NK cells both *in vitro* and *in vivo*[55], and seem to share precursors with ILC3s[56,57]. It is also now understood that NK cells are very heterogenous, and it is possible for different NK cell subsets to have distinct origins[58].

Circulating ILCPs have been identified in human peripheral blood defined by a $CD7^+$ $CD45RA^+$ $IL1R1^+$ $CD117^+$ surface phenotype[55]. These ILCPs share similarities with ILC3s but they are unable to produce cytokines. Extensive *in-vitro* culture (including single-cell cloning) using polarizing cytokines showed their ability to differentiate into mature NK cells, ILC1s, ILC2s and/or ILC3s. Their precursor activity was confirmed *in vivo* by the adoptive transfer into immunodeficient mice. Subsequent studies further characterized the differentiation potential of ILCPs. Expression of surface markers such as CD56, NKp46, KLRG1 and CD62L determines whether ILCPs are able to differentiate into ILC2s or into ILC3s and ILC1s/NK cells[56,57,59]. Furthermore, Notch signals promote RORγt expression and ILC effector function in human blood ILCPs[60].

ILCPs are not only found in the blood, but they are also present in various organs including lymph nodes[55,56]. This shows that, after entering tissues, ILCPs can give rise to mature ILC subsets, likely in response to local tissue-specific cues. These observations have led to the concept of local "ILC-poiesis" to generate diverse ILC subsets within organs[61]. However, the relative contribution of ILC recruitment versus local proliferation in the maintenance of mature ILCs in tissues is still unclear, especially in the context of tissue injury. It is also not known where human ILC proliferation takes place within tissues.

## 1.3 CELLULAR HETEROGENEITY OF ILCS

ILCs are phenotypically and functionally heterogenous in addition to being highly plastic. Single-cell RNA-sequencing has emerged as a technique for high-throughput analysis of transcriptomics in single cells, which allows unbiased investigation of heterogeneity between and within different cell subsets. In recent years, single-cell RNA-sequencing has been employed in the study of mouse[62–64] and human ILCs[20,29,62,65].

Crinier *et al.* performed single-cell RNA-sequencing of human and mouse NK cells isolated from blood and spleen. They identified species-specific and organ-specific transcriptional signatures, which suggests that splenic NK cells are more activated than their counterparts in the blood, based on the expression of effector genes, transcription factors (e.g., *JUN, KLF6, FOSB*), and genes encoding cell surface proteins (e.g., *KLRC1, ICAM1, CD69*)[62]. Furthermore, they identified three and four NK cell clusters in the mouse and human spleen, respectively. Two of these clusters were present in both spleen and blood, but one cluster from the mouse and two from human were unique and previously undefined[62]. This shows the power of single-cell RNA-sequencing for dissecting immune cell heterogeneity.

Björklund *et al.* established core and subset-specific transcriptional signatures of human ILCs from tonsils[29]. Among transcripts that are shared between all ILCs were *RARG* and *RORA*, confirming their role in ILC development and regulation. ILC1s expressed *IKZF3* and several other pathways to control their own activity, but they also expressed transcripts associated with T cells, such as *CD4, CD5, CD6, CD28, CD27* and *CCR7*. ILC2s expressed transcripts associated with response to, synthesis and breakdown of prostaglandins. This study also identified three distinct subsets of ILC3s: The first subset represented NKp44$^+$ ILC3s, a second smaller subset expressing *SELL* (CD62L) and CD45RA that represents an immature population later described as ILCPs[55], and finally, a third ILC3 subset expressing HLA-encoding transcripts that represents the human equivalent of mouse MHCII$^+$ ILC3s[66,67], later described as HLA-DR$^+$ ILC3s[68].

Another single-cell RNA-sequencing study of human ILCs from tonsil and colon revealed cellular intermediates in the plasticity from ILC3s to ILC1s[65]. In both organs Cella *et al.* identified several clusters of ILC3s and ILC1s, along with clusters co-expressing markers for ILC3 and ILC1 and a highly proliferative MKI67$^+$ expressing cluster. Trajectory analysis using RNA velocity indicated the direction of flow from the transitional clusters to ILC1.

Recently, a single-cell RNA-sequencing study of human ILCs from four organs (blood, tonsil, lungs, colon) has been published[20]. By comparing ILCs from 3 different immunological niches (circulation, mucosal tissue, lymphoid tissue), they identified transcriptional signatures and gene modules associated with each niche and organ. Genes controlling T lymphocyte recirculation, such as *S1PR1* and *SELL,* and integrins were preferentially expressed by ILCs in blood and tonsil. In contrast, ILCs from the lungs and colon expressed transcripts involved in the tissue residency of T lymphocytes. Naïve-like ILCs expressed a migratory signature in the blood but a tissue residency signature in the lung or colon. They also found ILC2 and ILC3 heterogeneity between and within different organs, with distinct transcriptional signatures involved in cell migration, tissue residency, and cell activation. Trajectory analysis revealed how the development of these subsets branched off from naïve-like ILCs. Finally, they also reported widespread TCR V(D)J-rearrangement in blood ILC1s, an unexpected result which reopens the discussion surrounding ILC1 identity.

In conclusion, the new technology of single-cell RNA-sequencing revealed that human ILCs are highly heterogenous and that ILCs have tissue-specific signatures.

## 1.4 ILC TISSUE DISTRIBUTION, TISSUE NICHES AND MIGRATION

In general, ILCs are rare cell in most organs. However, ILCs are more abundant in tissues that are constantly exposed to pathogens, such as the gut and lung[18,35]. The abundance of ILC subsets differs between tissues and organs, for example ILC3s are predominant in the intestine, whereasILC2s predominate in the skin and adipose tissue[69].

Studies using parabiotic mice established that, in steady state ILCs are mainly tissue-resident cells that are maintained by local self-renewal[70]. However, based on mouse studies, ILCs show motility within tissue and are able to traffic between organs in response to environmental signals. For example ILC3s can move in and out of cryptopatches in the intestine [71,72] and can patrol the inflamed intestinal barrier[73]. During infection or inflammation, ILCs are able to migrate from the circulation or from within the tissue to the site of infection[74–76]. In contrast to mice, circulating human ILCs exist in steady state. ILCs in human peripheral blood are mostly composed of mature ILC2s and a $CD117^{+}$ ILCP population[55,77]. ILCPs are also found in tissues, which suggests that these cells are also able to migrate in and out of the tissue and recirculate.

Mouse studies have shown that ILC migration is regulated by integrin and chemokine receptors specific for certain tissues and organs[69,78]. Expression of these receptors in ILCs generally mirrors expression in their T cell counterparts. For example, the integrin α4β7 and the chemokine receptor CCR9 are critical for the homing of ILCs to the small intestine[79]. Expression of both receptors by ILC3s and ILC1s are induced by retinoic acid, which adds a level of metabolic control to ILC migration.

Circulating ILCs and ILCPs are known to express CD62L, which is required for entry into the lymph nodes[80] via high endothelial venules. CCR7 is another receptor regulating ILC migration into lymph nodes[81]. On the other hand, ILC egress out of the lymph nodes occurs along a sphingosine-1-phosphate (S1P) gradient and is regulated by S1P receptors[74]. CD69 promotes prevents the egress and thereby the retention of lymphocytes by binding to S1PR1[82]. Accordingly, CD69 is expressed by tissue ILCs such as ILCs from the gut and skin[74,83,84]. Other mechanisms for tissue retention include integrins CD103 which binds to E-cadherin in the epithelia and CD49a which binds to collagen-IV. CD103 is expressed by several different human ILC subsets[65,85,86]. CD49a is expressed by mouse ILC1s and ILC3s[63], as well as by human intraepithelial ILC1s[86].

Not much is known about human ILC localization within organ-specific niches, and the factors determining human localization in relation to their function. This is in part due to the difficulty of studying human ILCs *in vivo*. The MISTRG humanized mouse model was used in this thesis to overcome this limitation and will be explained further in chapter 3.

## 1.5 ROLE OF LUNG ILCS DURING INFECTION AND INFLAMMATION

The lung is a complex organ specialized for gas exchange and therefore exposed to airborne challenges from the external environment. It is a common site for infection, chronic inflammatory diseases and cancer. Lung immune responses must be balanced with the need to main the lung's primary function (oxygen uptake). ILCs are uniquely positioned to play important roles in lung immunology. As their T cell counterparts, each ILC subset is specialized to respond to different challenges. ILC1s are specialized to respond to intracellular pathogens including viruses, ILC2s respond to helminths and parasites and ILC3s respond against extracellular bacteria and fungi. As mentioned before, the study of human ILCs is limited by the lack of *in-vivo* models for infection.

NK cells are important for defence against viral infections and cancer, and have been extensively studied[87,88]. Less is known about human lung ILCs during respiratory viral infections. Human ILCs are depleted from the circulation during HIV-1[89] and SARS-CoV-2 infection[90], but their role has not been investigated in the lung. Mouse studies have shown that ILC1s serve an important role as early responders during influenza infection by the rapid production of IFNγ[91,92]. As a more proinflammatory subset, ILC1s also contribute to inflammatory diseases. For example, conversion from ILC2s to ILC1s is observed during COPD [93] and is associated with exacerbation of the disease.

ILC2s play an important role in asthma and airway hyperresponsiveness, which has been extensively reviewed[94–98]. Asthma is mediated by type 2 cytokines, typically from Th2 cells, but evidence points out ILC2s as an early source of type 2 cytokines responsible for the initiation of eosinophilic inflammation in asthma[99]. In humans with asthma, ILC2s are increased in the blood and bronchoalveolar lavage (BAL) fluid[100–102], which correlates with impaired respiratory function[103,104]. Mouse studies have also shown that ILC2s interact with

and contribute to the activation of Th2 cells via MHCII[105] and OX40 ligand[106]. Additionally, lung ILC2s promote tissue repair following influenza infection via the production of amphiregulin[23].

Mouse studies demonstrate that the early production of IL-17 and IL-22 by ILC3s is important in the host response against bacterial pathogens such as *Klebsiella pneumoniae*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*[107–111]. IL-22-producing ILC3s are established early in the lung and confer protection shortly after birth, due to interaction with commensal microbiota, which stimulates the expansion and subsequent migration of ILC3s from the gut into the lung[44]. In *Mycobacterium tubercuosis* infection, ILC3s are crucial for the early recruitment of macrophages and the formation of protective lymphoid follicles and granulomas via the induction of CXCL13[112]. Finally, ILC3s can also provide trained immunity against *Citrobacter rodentium* in mice[113].

In patients with various infectious and inflammatory lung diseases, ILCs are depleted from the peripheral blood, suggesting that they migrate into sites of inflammation. However, not much is known about how human ILCs migrate *in vivo*. Targeting ILC migration could be useful to develop treatment for human lung diseases.

# 2 RESEARCH AIMS

The overarching aim of this thesis work is to investigate *in vivo* how the spatial distribution of human ILCs determine their characteristics and function, with a focus on lung ILCs. Specific aims of each study within this thesis are as follows:

- To determine the ontogeny, localization and function of $CD5^+$ human lung ILCs **(Paper I)**
- To map sites of proliferation of human ILCs across different organs and tissue compartments **(Paper II)**
- To define the molecular signatures of human ILCs and NK cells in the vascular and tissue niches in the lung **(Paper III)**

# 3 MATERIALS AND METHODS

## 3.1 MISTRG HUMANIZED MOUSE MODEL

Studies using animal models are not always relevant to humans due to differences in physiology, on the other hand *in vivo* studies on humans are very limited due to ethical and practical reasons. Humanized mouse models are created to address this problem, by having the advantages of an *in vivo* experimental system in a small animal model while still producing findings that are translatable to humans. In general, humanized mice are mice with a human immune system, generated by transplanting human cells or tissues into immunodeficient mice. Further details on different humanized mouse models and their contribution to discoveries in macrophage and innate lymphoid cell biology have been previously reviewed[114–116].

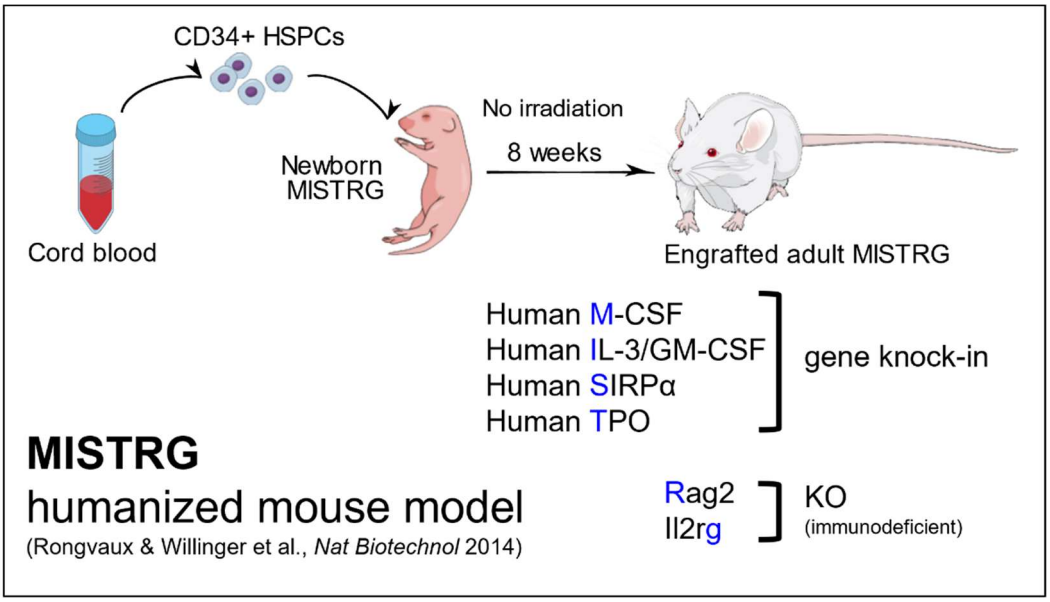


**Figure 2.** The MISTRG humanized mouse model, generated from an immunodeficient *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ background with several human gene knock-ins and engrafted with human CD34$^{+}$ HSPCs from cord blood. Figure designed with Mind the Graph and adapted from Paper I, Fig. 1.

The MISTRG humanized mouse model[117] is the *in-vivo* experimental model used for all three papers discussed in this thesis. MISTRG mice are made immunodeficient by knocking out two genes: *Rag2* and *Il2rg*. *Rag2* is required for the assembly of antigen receptors in B cells and T cells, therefore *Rag2* deficiency blocks the development of both B and T cells. *Il2rg* encodes the common gamma chain of the IL-2 receptor, which is required, among others, for IL-15 signaling. IL-15 is essential for the development and homeostasis of NK cells and therefore *Il2rg*$^{-/-}$ mice lack mouse NK cells. With both *Rag2* and *Il2rg* knocked out, MISTRG mice lack mouse T cells, B cells, and NK cells, which prevents the rejection of transplanted human cells by mouse lymphocytes.

In addition to lacking mouse *Rag2* and *Il2rg*, MISTRG mice express the human cytokines macrophage colony-stimulating factor (M-CSF), IL-3, granulocyte-macrophage

colony-stimulating factor (GM-CSF), and thrombopoietin (TPO) through gene knock-in[117]. M-CSF and GM-CSF are two important cytokines for the development and maintenance of monocytes and macrophages. IL-3 supports the proliferation of early HSPCs. TPO is required for the self-renewal and long-term maintenance of HSPCs. Expression of human cytokines in MISTRG mice is achieved by the knock-in of the human genes into the respective mouse loci[118]. In homozygous knock-in mice both alleles of the mouse gene are completely replaced by the human gene. With this method, MISTRG mice can achieve physiological levels of human M-CSF, IL-3, GM-CSF, and TPO[117,119–122] while not expressing the mouse counterparts therefore giving a competitive advantage for human hematopoiesis. All these factors create a highly permissive environment for human hematopoiesis and favor the development of human myeloid compartment[117,123–125]. In turn, the presence of human myeloid cells supports the development of human NK cells through the trans-presentation of human IL-15[117].

The MISTRG model we use also includes the human *signal regulatory protein alpha* (*SIRPA*) gene knocked in[117]. SIRPα suppresses phagocytosis in phagocytic cells such as macrophages following interaction with lymphocytes expressing CD47. This interaction is species-specific, as mouse and human SIRPα and CD47 do not recognize one another. Expression of human *SIRPA* in residual mouse myeloid cells through gene knock-in prevents human $CD47^+$ lymphocytes from being phagocytosed in MISTRG mice. This improves the survival of human lymphocytes in the MISTRG mouse and allows high levels of engraftment with human hematopoietic cells[117,122].

To generate the humanized mice, human $CD34^+$ HSPCs isolated from cord blood were transplanted into newborn (2-3 days old) MISTRG mice by intrahepatic injection. The mouse liver is still an active hematopoietic organ around the time of birth, which is a suitable environment for the expansion and development of the human HSPCs into a functioning human immune system. At 6-7 weeks of age blood was taken from the mice to check for engraftment of human $CD45^+$ hematopoietic cells. All major human immune cell subsets are present in HSPC-engrafted MISTRG mice 7 weeks after transplantation. Various organs (lungs, liver, spleen, bone marrow, peripheral blood) were collected from 2-3 months old engrafted MISTRG mice and constitute the main *in-vivo* sample source for experiments in all three papers.

Humanized mouse models typically require a preconditioning step to clear the mouse bone marrow hematopoietic niche and provide space for the transplanted human $CD34^+$ HSPCs to differentiate and expand. This is usually achieved by sub-lethal irradiation using X-ray or $^{137}Cs$. The MISTRG mouse model does not require preconditioning due to already having a "genetic preconditioning" in the form of human cytokine knock-ins. This is advantageous because it removes the potential bias introduced by the damaging effects of irradiation. MISTRG mice used in this work were not irradiated except for mice used for single-cell RNA-sequencing studies, which were irradiated with 100 cGy from $^{137}Cs$ prior to transplantation.

Several limitations of the MISTRG mouse model and humanized mouse models in general include the lack of human fetal hematopoiesis, thus the absence of ILCs derived from

fetal progenitors in the intestine. Lymph nodes other than the spleen are not fully developed, which may alter the migration and proliferation of human ILCs in MISTRG mice. Despite the limitations, the MISTRG humanized mouse model could provide important information on human ILCs *in vivo* beyond what is possible from *ex vivo* studies on human blood and tissues.

## 3.2 HUMAN SAMPLES

Umbilical cord blood is the main source of human $CD34^+$ HSPCs used to generate MISTRG mice with a human immune system. Umbilical cord blood was obtained from caesarian sections at the Karolinska University Hospital, Huddinge. $CD34^+$ HSPCs were isolated from cord blood by positive selection using a bead-based immunomagnetic cell separation method. Isolated $CD34^+$ HSPCs were stored frozen and thawed as needed on the day of transplantation.

Buffy coats were obtained from the Blood Bank at the Karolinska University Hospital, Huddinge. As part of the IHOPE study, human lung and spleen tissue were obtained through the Transplantation Clinic at Karolinska University Hospital, Huddinge from deceased organ donors whose organs were not used for transplantation.

## 3.3 FLOW CYTOMETRY AND CELL SORTING

### 3.3.1 Principle

Flow cytometry is a fundamental technique in immunology where multiple parameters such as size and fluorescent properties of single cells can be measured simultaneously[126]. This is achieved by creating a single cell stream where the cells are passed through an array of lasers and analyzed for light scatter and fluorescence. Tissue must first be digested to create a single-cell suspension of immune cells. The cells are then stained with a panel of fluorochrome-conjugated antibodies against surface or intracellular proteins before running them through the flow cytometer. Using this technique, cell populations can be identified and characterized by phenotypic markers specifically expressed by them.

One advantage of flow cytometry is the that the cells are kept alive during the process, so a variant of this method can be done where specific cell populations are sorted for downstream experiments, such as for *in-vitro* culture or single-cell RNA-sequencing.

### 3.3.2 Analysis

The definition of ILCs as ‘lineage negative’ necessitates creating a panel of markers and a gating strategy to exclude cells expressing these lineage markers. It is especially important to distinguish them from T cells, and for this purpose antibodies against CD3 and TCRαβ are used. NK cells are distinguished from helper ILCs by the expression of CD94. Other lineage markers include CD11c and CD123 for dendritic cells, CD14 for monocytes, CD19 for B cells, CD34 for hematopoietic stem cells and FcεRI for mast cells and basophils. Human ILCs themselves are characterized by the expression of the IL-7 receptor α-chain (CD127) and

further subdivided into three subsets based on the expression of CRTH2 and c-Kit (CD117). The gating strategy for analysis on FlowJo is depicted in Figure 3.

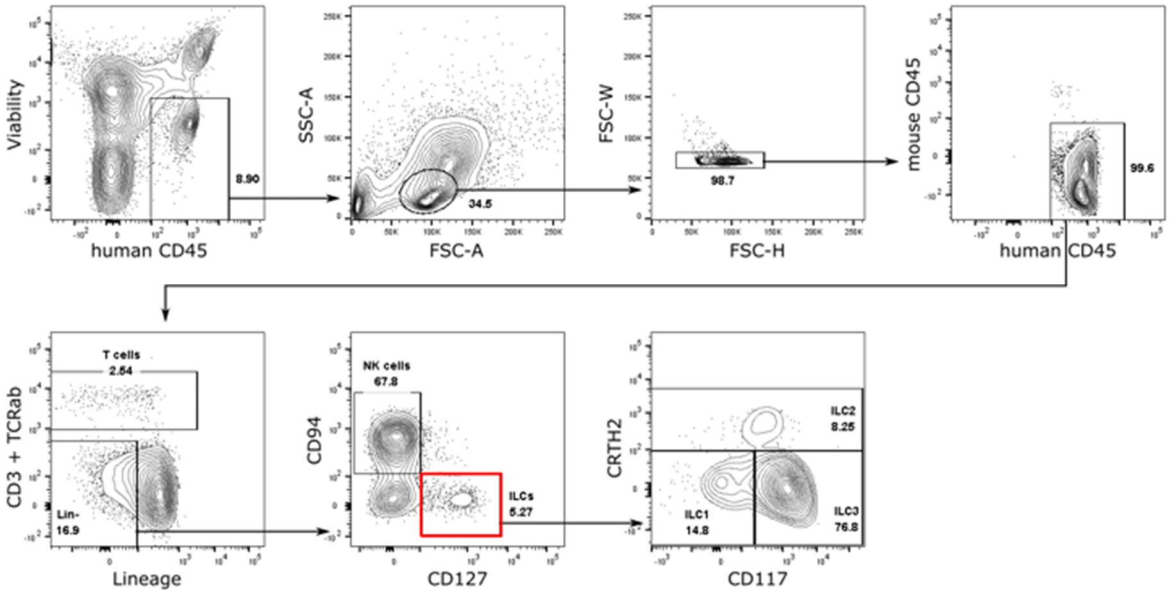


**Figure 3.** Gating strategy for flow cytometry analysis of human lung ILCs from HSPC-engrafted MISTRG mice. Human ILCs are gated as viable human $CD45^+$ $CD3^-$ $TCR\alpha\beta^-$ $CD94^-$ $Lineage^-$ $CD127^+$ lymphocytes that are further subdivided into ILC1s ($CRTH2^-$ $CD117^-$), ILC2s ($CRTH2^+$) and ILC3s ($CRTH2^-$ $CD117^+$). Lineage markers are CD11c, CD14, CD19, CD123, CD34 and FcεRI. Adapted from Paper I, Fig. 1.

Transcription factors and intracellular cytokine staining combined with cell permeabilization techniques were also used to further characterize the cells and investigate their functional or maturation status. Mature ILC1s (and NK cells) express the signature transcription factor T-bet and produce the cytokine IFNγ upon stimulation. ILC2s express the signature transcription factor GATA3 and produce several cytokines, among them IL-13. ILC3s express the signature transcription factor RORγt and produce the cytokines IL-17 and IL-22. Two transcription factors that are known to play a role in ILC development were also used in Paper II: PLZF which regulates the development of early ILC progenitors[46] and TCF-1 which regulates stemness in lymphocytes[127].

Finally, several methods could be employed to assess the proliferation status of the cell. For example, staining for the nuclear protein Ki67 is commonly used to identify proliferating cells. A DNA binding dye such as propidium iodide (PI) can be incorporated to investigate specific stages of the cell cycle.

## 3.4 INTRAVASCULAR CELL LABELING

Tissues within organs are spatially divided into compartments, with each compartment having a different function. For example, the lung is divided into three compartments: the vasculature, the interstitial lung tissue, and the airways. The compartment in which a cell is

located can give important clues about its function. However, in the process of isolating cells from tissues for flow cytometry, the tissue is digested and thus the spatial information is lost. Intravascular cell labeling is a technique used in mouse studies in which immune cells in the vascular compartment of the mouse are labelled an intravenously injected and fluorochrome-conjugated antibody[128], preserving the spatial information of the two compartments (vascular and tissue) prior to digestion. In HSPC-engrafted MISTRG mice, an anti-human CD45 antibody conjugated with PE or PE-Cy7 was used. The antibody was injected intravenously into the mice to label cells within the vasculature and the mice were sacrificed 5 minutes after antibody injection. Organs were harvested to isolate cells, which were then counterstained with an anti-human CD45 antibody conjugated with a different fluorochrome (in this case, APC-Cy7) together with antibodies against other surface markers to define ILCs and NK cells Schematic representation of this technique is depicted in Figure 4.

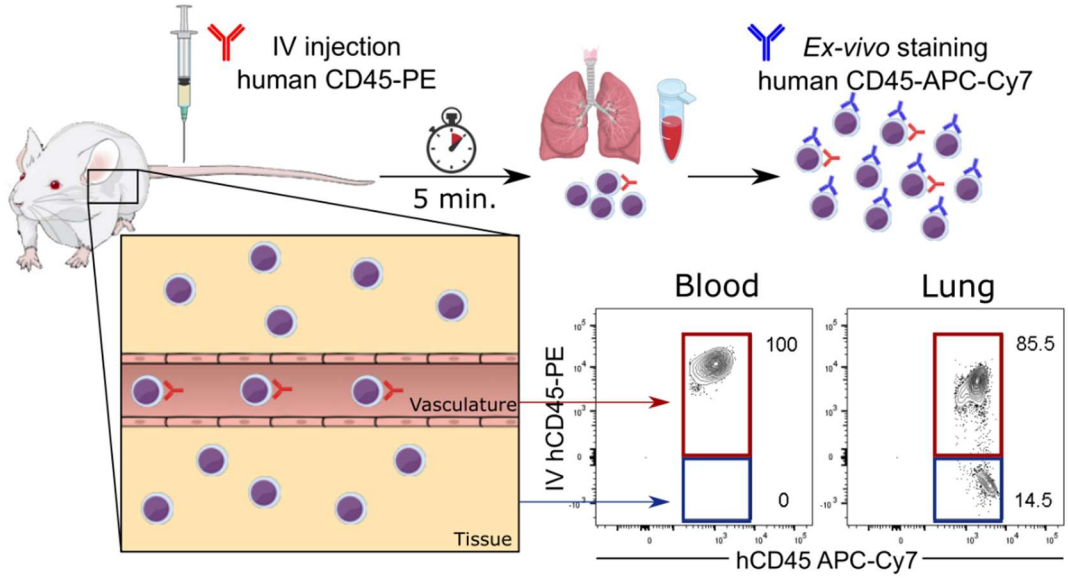


**Figure 4.** Intravascular cell labeling technique. HSPC-engrafted MISTRG mice were injected intravenously (IV) with the anti-human CD45-PE antibody (red) and sacrificed after 5 minutes. Cells isolated from organs were stained *ex-vivo* with CD45-APC-Cy7 (blue). Figure was designed with Mind the Graph and adapted from Paper I, Fig. 5.

To achieve a clear separation between vascular and tissue populations, the amount of hCD45-PE antibody injected into the mice must be saturating but not excessive. Figure 5. depicts the antibody titration graph. The final amount of IV-CD45-PE antibody used in all three studies was 2 µg per mice.

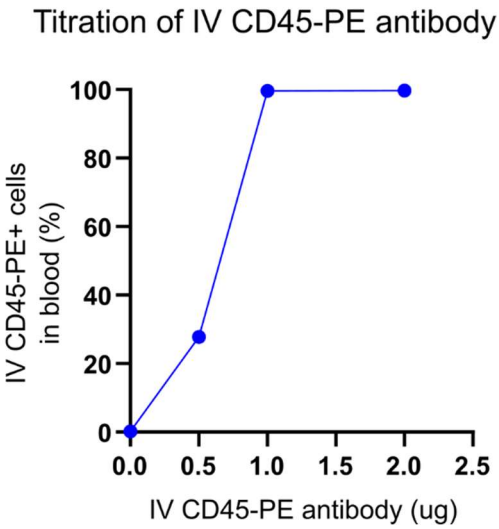


**Figure 5.** Titration of the intravenously (IV) injected hCD45-PE antibody measured as IV CD45-PE$^{+}$ human cells in the blood of HSPC-engrafted MISTRG mice 5 minutes after antibody injection. Figure was adapted from Paper III, Fig. 1.

Flow cytometric analysis showed two distinct populations when gating on IV hCD45-PE and hCD45 APC-Cy7 (see Figure 6). Nucleated human hematopoietic cells in the blood and within the vascular compartment of organs will be double positive for IV hCD45-PE and hCD45 APC-Cy7. Cells within the tissue or other compartments not in contact with blood will only be positive for hCD45 APC-Cy7.

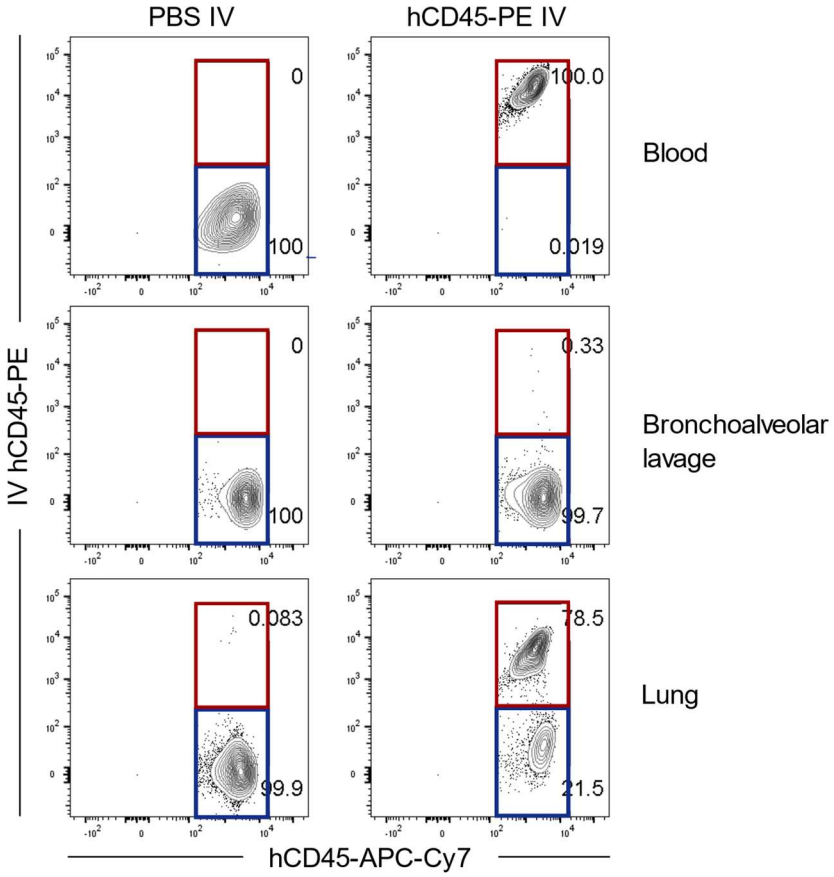

**Figure 6.** Intravascular labeling results of human cells in peripheral blood, BAL and lung from HSPC-engrafted MISTRG along with PBS controls. Cells isolated from the blood are completely stained with the IV hCD45-PE antibody, whereas cells in the airways (BAL) are not stained. Cells from the lung are divided into two populations based on intravascular labeling which corresponds to cells in the vascular and tissue compartments. Figure was adapted from Paper I, Fig. S4.

## 3.5 IN-VITRO CULTURE

OP9-DL1 is a cell line derived from mouse bone marrow stromal cells expressing the human Notch ligand Delta-like ligand 1 (DLL1). The Notch signaling pathway is highly conserved and regulates cell differentiation, including lymphocyte development. In general, mammals possess four different Notch receptors (Notch 1-4) and five different Notch ligands (DLL1, DLL3, DLL4, Jag1, Jag2). The OP9-DL1 cell line was originally developed by Zúñiga-Pflücker's group to study T cell lineage commitment and differentiation *in vitro*[129]. However, there has been increasing evidence that Notch signaling is also important for the differentiation, survival, and proliferation of ILCs. OP9-DL1 and OP9-DL4 have now been used as a model to study ILC differentiation *in vitro*[42,55–57,59], although it is important to note that *in-vitro* systems are not always representative of *in-vivo* conditions.

In Paper I, the differentiation potential of $CD5^+$ ILCs was investigated using the OP9-DL1 culture system. ILCs were bulk sorted from blood and then co-cultured with OP9-DL1 cells and a combination of cytokines for polarizing ILCs into specific ILC subsets. These cytokines were IL-1β and IL-12 for ILC1s; IL-4, IL-25, IL-33 and TSLP for ILC2s and IL-1β and IL-23 for ILC3s.

## 3.6 SINGLE-CELL RNA-SEQUENCING

Transcriptional profiling allows to view a snapshot of genes that are transcribed by cells in a sample at one point in time. With the advent of single-cell RNA-sequencing, transcriptional profiling of individual cells has become a powerful tool to resolve cellular heterogeneity within samples[130–132]. There are several different methods for single-cell RNA-sequencing, among them are single cell sorting-based and droplet-based platforms. In the droplet-based method (10x Genomics), individual cells in the sample are partitioned into droplets containing reagents for cDNA synthesis and a unique barcode. After sequencing, individual cell transcriptomes are reconstructed using computational tools. Further analysis using bioinformatic tools allows us to identify clusters of cells based on transcriptional similarity. In addition, analysis of cluster-specific differentially expressed genes allows defining discrete cell subsets and their functional properties.

Single-cell RNA-sequencing is a powerful tool for identifying novel cell populations, but it is important to note that transcription of a gene does not always result in translation into a protein. Algorithms used in the analysis of single-cell RNA-sequencing data also create clusters using predefined settings. Therefore, follow-up experiments need to be done to confirm the identity and function of cell populations.

## 3.7 STATISTICAL ANALYSIS

Results were obtained from at least two independent experiments. For comparison between two groups, we used Student's *t* test to determine statistical significance. For comparison between more than two groups, one-way ANOVA was used followed by *post hoc* testing using Tukey's multiple comparison test. Statistical analysis was performed in GraphPad Prism 8.

## 3.8 ETHICAL CONSIDERATIONS

### 3.8.1 Mouse experiments

This thesis studied the spatial distribution of human ILCs *in vivo* but performing invasive *in vivo* experiments on humans is ethically unacceptable. Furthermore, access to human tissues is limited and spatial information is generally lost during the process of cell isolation. *In-vitro* culture techniques also provide limited information regarding spatial distribution and are not fully representative of *in-vivo* conditions. The MISTRG humanized mouse model was chosen to overcome these limitations and was therefore necessary for achieving the aims of this thesis.

The use of mice in scientific experiments in general can provide us with valuable information, but since it can also potentially cause pain and suffering it is important that stringent requirements regarding animal welfare and experimental design are met. The use of the MISTRG mouse model in our studies was reviewed and approved by the Linköping Animal Experimentation Ethics Committee. All experiments using the MISTRG mice were designed and performed according to the 3R principles (replacement, reduction, refinement). Experiments were discussed and carefully planned, the number of animals used was kept to a minimum and any treatments that might cause potential suffering were revised to reduce the effects on the animals. Daily monitoring and supervision of all animals by the animal facility staff and laboratory technician were done to ensure their welfare.

MISTRG mice have been thoroughly characterized[117]. Non-engrafted MISTRG mice generally have a long lifespan (> 9 months) even though they eventually develop pulmonary alveolar proteinosis syndrome due to a lack of functional alveolar macrophages[117,123–125]. However, high engraftment of MISTRG mice with human HSPCs can lead to anemia after 4-5 months when human hematopoiesis takes over the bone marrow, resulting inefficient mouse erythropoiesis. Therefore, all experiments using HSPC-engrafted MISTRG mice were performed and concluded prior to 2-3 months post-engraftment, before the mice developed anemia. Anemia in MISTRG mice correlates with levels of engraftment with human hematopoietic cells, which could be adjusted by reducing the number of human HSPCs transplanted into the mice. In this way, the potential suffering due to anemia in MISTRG mice can be reduced.

The use of animals in scientific experiments has always been a sensitive subject and therefore it is an important matter to discuss and reflect on. However, the potential benefits of

the research in this thesis towards understanding an important aspect of human ILC biology and the therapeutic applications justifies its use.

### 3.8.2 Human samples

Umbilical cord blood is one of the least problematic sources of human $CD34^+$ HSPCs, which is required for generating humanized MISTRG mice. Umbilical cord blood was collected from routine caesarean sections and the collection is a non-invasive procedure which does not pose any risk or distress to the mother or the newborn child. The parents were informed that the cord blood will be used for research to study the human immune system using humanized mice and they gave their informed consent. Furthermore, the use of cord blood was approved by the Ethical Review Board at Karolinska Institutet.

Several other human samples were used. Buffy coats from the Blood Bank at Karolinska Institutet were donated by healthy donors after obtaining their informed consent. Paper I also used human lung and spleen tissue samples, which were obtained from deceased organ donors (as part of the IHOPE study at CIM) when the organs were not used for transplantation. Informed consent was obtained for all donors, the study was performed in accordance with the Declaration of Helsinki and approved by the Ethical Review Board at Karolinska Institutet.

Finally, genetic data obtained from the single-cell RNA-sequencing experiments of MISTRG mice engrafted with cord blood HSPCs are considered sensitive personal data. Therefore, the sequencing data were stored and processed according to the General Data Protection Regulation (GDPR) and Swedish legislation. The donors explicitly consented that the donated umbilical cord blood could be used for RNA-sequencing.

# 4 RESULTS AND DISCUSSION

## 4.1 THE ONTOGENY, LOCALIZATION, AND FUNCTION OF CD5$^+$ HUMAN LUNG ILCS

CD5 is a surface protein found on T lymphocytes and at lower amounts on a subset of B lymphocytes. Consequently, in several human ILC studies CD5 has been included in a panel of markers to exclude T cells. However, since then it has been established that ILC1s also express CD5 as well as other T cell surface proteins[29,31,37]. Furthermore, CD5-expressing ILCs have been found in human peripheral blood, umbilical cord blood and thymus[32,33,133]. In addition, CD5$^+$ ILCs are able to differentiate into mature ILC2s and KIR$^+$NKG2A$^-$ NK cells, at least *in vitro*[32,33]. These observations are consistent with CD5$^+$ ILCs being heterogenous and having distinct features than conventional CD5$^-$ ILCs. In Paper I, we dissected the ontogeny, localization, and function of CD5$^+$ human lung ILCs *in vivo*.

### 4.1.1 CD5$^+$ ILCs occupy the intravascular niche of the lung and other organs, and stay in the circulation at steady state

CD5-expressing ILCs were present both in human lung samples and lungs of human HSPC-engrafted MISTRG mice (Paper I, Fig. 1C-D), but they were most abundant in peripheral blood (Paper I, Fig. 1E). This suggests that CD5$^+$ ILCs reside in a different anatomical compartment within organs compared to conventional CD5$^-$ ILCs. Intravascular cell labeling with the human CD45-PE antibody demonstrated that CD5$^+$ ILCs predominantly resided in the lung vasculature of HSPC-engrafted MISTRG mice, similar to NK cells (Paper I, Fig. 5B). Conventional CD5$^-$ ILCs were equally distributed between the vascular and tissue compartment of the MISTRG lung. This was corroborated by the low expression of the tissue residency markers CD69 and CD103 in CD5$^+$ ILCs compared to CD5$^-$ ILCs. CD5$^+$ ILCs instead express more CD62L, which is a marker that mediates lymphocyte recirculation and their homing to secondary lymphoid organs (Paper I, Fig. 5D). Overall, we showed that CD5$^+$ ILCs reside in the intravascular niche together with NK cells and could possibly recirculate to secondary lymphoid organs at steady state.

### 4.1.2 CD5$^+$ ILCs are a heterogenous population containing ILC1s and an immature ILC population

Further investigation of the identity and function of CD5$^+$ ILCs revealed that they are a heterogenous population. The CD5$^+$ ILC population includes mature ILC1s which are characterized by the CD117$^-$CRTH2$^-$ surface phenotype (Paper I, Fig. 2A), expression of the ILC1 lineage-defining transcription factor T-BET and correspondingly the lack of GATA3 and RORγt (Paper I, Fig. 2B). Following PMA and ionomycin stimulation *in vitro*, CD5$^+$ ILCs produced the signature ILC1 effector cytokine IFNγ and to a lesser extent the ILC2 cytokine IL-13, whereas they did not produce the ILC3 cytokines IL-22 and IL-17A (Paper II, Fig. 2C-D). However, the majority of CD5$^+$ ILCs did not produce any of the signature cytokines which suggested that they might be immature. This was further supported by the surface expression of CD45RA, a marker for naïve T cells and ILCPs, intermediate CD127 expression and the

lack of the mature ILC marker HLA-DR (Paper I, Fig. 3A-B). $CD5^+$ ILCs also expressed variable amounts of transcription factors involved in ILC development such as PLZF, E4BP4, and especially TCF-1 (Paper I, Fig. 3D). Finally, $CD5^+$ ILCs contained a subset of actively dividing cells as shown by staining for the proliferation marker Ki67 (Paper I, Fig 3C). Taken together, $CD5^+$ ILCs are heterogenous and contain a mixture of mature ILC1s and an immature, actively dividing population at steady state.

### 4.1.3 $CD5^+$ ILCs downregulate CD5 in polarizing conditions and acquire effector functions

Our results showed that $CD5^+$ ILCs contain an immature population. To investigate their differentiation potential, we used an *in-vitro* OP9-DL1 co-culture system, where sorted $CD5^+$ ILCs and $CD5^-$ ILCs were cultured with lineage-specific polarizing cytokines. $CD5^-$ ILCs contained mature ILCs along with $CD117^+$ ILCPs which can differentiate to all ILC subsets under polarizing conditions, which was corroborated with our results. On the other hand, $CD5^+$ ILCs contained mature ILC1s and some ILC2s, but could similarly produce all three ILC subsets, with IFNγ-producing ILC1s predominating (Paper I, Fig. 4A-B). Interestingly, the majority of $IFN\gamma^+$ ILCs cultured from sorted $CD5^+$ ILCs downregulated their CD5 surface expression, while the ones still expressing CD5 consisted of both $IFN\gamma^+$ ILCs and non-producing cells (Paper I, Fig. 4C). This demonstrates that under polarizing conditions, $CD5^+$ ILCs acquire effector functions and downregulate CD5, which shows that CD5 might inhibit the effector function in immature ILCs.

### 4.1.4 $CD5^+$ ILCs have a distinct ontogeny and represent an alternative pathway of development

The distinct localization of $CD5^+$ ILCs within the vasculature suggested that they could have a distinct origin, which we investigated by examining the reconstitution of human ILCs in MISTRG mice at 3, 5, and 8-11 weeks after transplantation with human HSPCs. Human ILCs already appeared in the bone marrow, liver, and spleen at 3 weeks post transplantation, while very few were present in the lung (Paper I, Fig. 6A-B). ILC reconstitution occurred before the expansion of NK cells and T cells (Paper I, Fig. S5). Contrary to $CD5^-$ ILCs that have a bone marrow origin, $CD5^+$ ILCs already resided in the liver, spleen (Paper I, Fig. 6A-B) and thymus (Paper I, Fig. 6C) at 3 weeks post-HSPC transplantation. $CD5^+$ ILCs were found in both the vascular and tissue compartment of adult HSPC-engrafted MISTRG, suggesting that they develop from human $CD34^+$ HSPCs that entered and resided in the spleen and liver before gaining access to the vasculature.

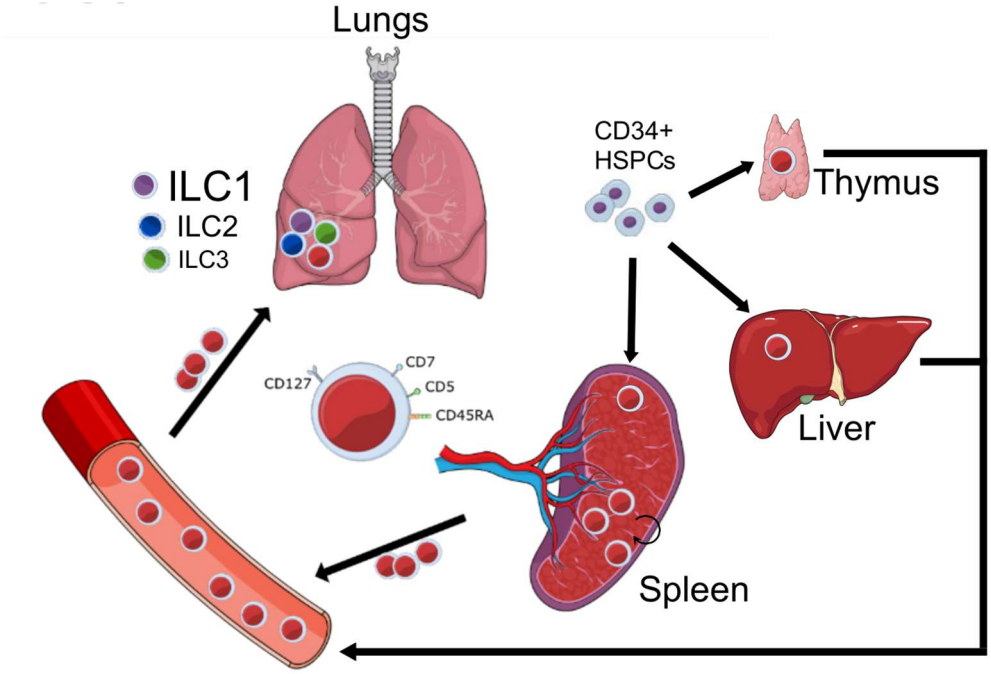


**Figure 7.** Graphical summary of Paper I

## 4.2 MAPPING SITES OF PROLIFERATION OF HUMAN ILCS ACROSS DIFFERENT ORGANS AND TISSUE COMPARTMENTS

As first responders in host defense and guardians of organ homeostasis, ILCs are mainly tissue-resident and quiescent cells in steady state[70], but they are able to expand rapidly in response to cytokines that are induced by tissue injury. ILCs also exist in the circulation, and they migrate during their development and later in life to inflamed organs[17,71,72,75,76,79,81,134,135]. However, how human ILCs are compartmentalized between the vasculature and the parenchyma within organs is poorly understood. The relationship between human ILC localization and proliferation is also unknown. In Paper II, we mapped sites of proliferation of human ILCs across different organs and tissue compartments using the MISTRG humanized mouse model.

### 4.2.1 The proliferative state of human ILCs varies according to subsets and spatial compartments within tissues

By performing intravascular cell labeling and subsequently staining cells isolated from four different organs for the proliferation marker Ki67, we are able to determine the spatial distribution of quiescent and proliferative ILCs in HSPC-engrafted MISTRG mice (Paper II, Fig. 2A). Four different ILC populations were identified: quiescent tissue-resident (Q-R), quiescent intravascular (Q-IV), proliferative tissue-resident (P-R), proliferative intravascular (P-IV). The relative frequency of these populations differed among ILC subsets in the different organs. ILC2s, ILC3s and CD117$^{+}$ ILCPs were mostly quiescent in the spleen, lung and liver with ILC2s being predominantly intravascular in these organs. ILC3s and CD117$^{+}$ ILCPs were found in the vasculature of the lung and liver but were more tissue-resident in the spleen. CRTH2$^{-}$ CD117$^{-}$ ILC1s were found to be more proliferative than ILC2s and ILC3s, especially in the spleen and were found in the local vasculature of the spleen, lung and liver (Paper II, Fig.

2B-C). Taken together, our results showed that human ILCs subsets reside in distinct spatial compartments and have different proliferative states in different organs.

### 4.2.2 Highly proliferative immature CD45RA$^+$ CRTH2$^-$ CD117$^-$ ILC population express TCF-1 and reside in the spleen

Further investigation into the highly proliferative CRTH2$^-$ CD117$^-$ "ILC1" population revealed that they mostly expressed CD45RA, a marker for naïve T cells, immature ILCs and ILCPs (Paper II, Fig. 3A). Ki67$^+$ CD45RA$^+$ CRTH2$^-$ CD117$^-$ ILCs were found both in the intravascular and tissue compartment of the spleen and they had a predominantly intravascular localization in the lung and liver (Paper II, Fig. 3B-C). In the spleen, the proliferative CD45RA$^+$ CRTH2$^-$ CD117$^-$ ILCs were found to be in the G1 phase of the cell cycle. Among ILC lineage-defining transcription factors we examined, CD45RA$^+$ CRTH2$^-$ CD117$^-$ ILCs neither express EOMES, RORγt nor T-BET (Paper II, Fig. 4A), therefore excluding the possibility that they are mature NK cells, ILC3s or ILC1s, respectively. Interestingly, CD45RA$^+$ CRTH2$^-$ CD117$^-$ ILCs expressed the transcription factor TCF-1 (Paper II, Fig. 4B), which is associated with stemness and proliferation[127]. TCF-1 was also expressed by CD117$^+$ CRTH2$^+$ ILCPs/ILC3s but only few were Ki67$^+$, whereas CD45RA$^+$ CRTH2$^-$ CD117$^-$ ILCs were Ki67$^+$ and expressed high TCF-1. When stimulated *in vitro* with PMA and ionomycin, CD45RA$^+$ CRTH2$^-$ CD117$^-$ ILCs from engrafted MISTRG mice and human umbilical cord blood did not produce IFNγ, further confirming that this population are not mature ILC1s (Paper II, Fig 4C). In conclusion, proliferative CRTH2$^-$ CD117$^-$ ILCs resided in both the vascular and tissue niche of the spleen, expressed TCF-1 and had a phenotype and functional characteristics of immature ILCs.

### 4.2.3 CRTH2$^-$ CD117$^-$ CD45RA$^+$ ILCs have a distinct ontogeny

The CRTH2$^-$ CD117$^-$ CD45RA$^+$ ILC population was also present in human umbilical cord blood (Paper II, Fig. 5A-C), suggesting their possible role in seeding the organs during development. We investigated the development of CRTH2$^-$ CD117$^-$ CD45RA$^+$ ILCs by examining the kinetics of human ILC reconstitution following transplantation of MISTRG mice with human HSPCs. CRTH2$^-$ CD117$^-$ ILCs were the prevalent subset in the spleen, liver, and lung at 3 weeks post-transplantation (Paper II, Fig. 6A-B) and inhabited a radio-insensitive developmental niche (Paper II, Fig. S4A-B). The frequency of CD45RA$^+$ cells within the CRTH2$^-$ CD117$^-$ ILC subset in the spleen decreased over time (Paper II, Fig. 6C-D), which suggests their potential differentiation into CD45RA$^-$ CRTH2$^-$ CD117$^-$ ILCs. We conclude that CD45RA$^+$ CRTH2$^-$ CD117$^-$ ILCs have a distinct ontogeny and may help expand human ILCs in early life.

### 4.2.4 Single-cell RNA-sequencing reveals the heterogeneity of vascular and tissue ILCs in the spleen

Next, to determine the molecular profiles of human ILCs in the intra- versus the extravascular space of the spleen, we performed single-cell RNA-sequencing of sorted splenic ILCs from pooled cells isolated from HSPC-engrafted MISTRG mice, using the 10x Genomics platform. Intravascular cell labeling with the IV anti-human CD45-PE antibody was performed

to sort ILCs from the intravascular and extravascular space of the spleen. After the removal of contaminating cells, unsupervised clustering resulted in 8 lymphocyte clusters (Paper II, Fig. 7B), of which 6 were ILCs. Cluster 4 was identified as the ILC1/NK cell cluster, which was predominantly intravascular (Paper II, Fig. 7C) and characterized by the expression of the chemokine *CCL5* and lytic granule molecules (*GZMK, GNLY, NKG7*) (Paper II, Fig. 7D). Cluster 6 was present both in the vascular and tissue compartment (Paper II, Fig. 7C) of the spleen and expressed ILC2 signature genes, such as *GATA3, KLRB1, KLRG1, HPGD* and *HPGDS* (Paper II, Fig. 7E). The two ILC3 occupied distinct anatomical niches in the spleen. Cluster 0 was enriched in the intravascular space (Paper II, Fig. 7C) and expressed ILC3 genes associated with the spleen and secondary lymphoid organs (such as *TNFRSF25*, *TYROBP*, *NFKBIA* and *ZFP36*) as well as cell migration genes such as *ITGB1*, *CD44*, and genes encoding the S100A proteins (Paper II, Fig. 7F). The second ILC3 cluster, cluster 1, resided in the extravascular compartment (Paper II, Fig. 7C) of the spleen and expressed the ILC3 genes *ID2*, *LTB* and *TNFRSF4* that are important for lymphoid tissue formation (Paper II, Fig. 7F), along with other ILC3 genes associated with secondary lymphoid organs (*B2M*, *HLA-B*, *HLA-C*, *TNFRSF18*, *TYROBP*, *IFITM1*, *IFITM2*).

Two ILC clusters were found that did not correspond to mature NK cell and ILC subsets. ILCs in cluster 3 were found more in the vasculature than in the tissue compartment (Paper II, Fig. 7C) and expressed *ZBTB16* (Paper II, Fig. 7G), which encodes the transcription factor PLZF required for ILC development. Cluster 3 ILCs otherwise expressed very few distinctive genes, therefore these ZBTB16-ILCs could correspond to an intermediate cellular state of ILC differentiation. Cluster 2 ILCs were present in both the vascular and tissue compartment (Paper II, Fig. 7C) of the spleen and transcribed genes promoting proliferation (*MKI67*, *TOP2A*, *PCNA, STMN1*) (Paper II, Fig. 8A). Their proliferative gene signature was confirmed by Gene Ontology over-representation analysis (Paper II, Fig. 8B) and cell cycle scoring showed that most cluster 2 ILCs were in the S or G2/M phase (Paper II, Fig. 8C). MKI67-ILCs also expressed *TCF7* (encoding TCF-1) (Paper II, Fig. S5B-C) consistent with flow cytometry results (Paper II, Fig. 4B), and several transcription factors that control lymphocyte proliferation (Paper II, Fig. 8D). In conclusion, our single-cell RNA-sequencing results showed that mature human ILC subsets reside in the spleen, along with a separate cluster of proliferative ILCs consistent with our flow cytometry results.

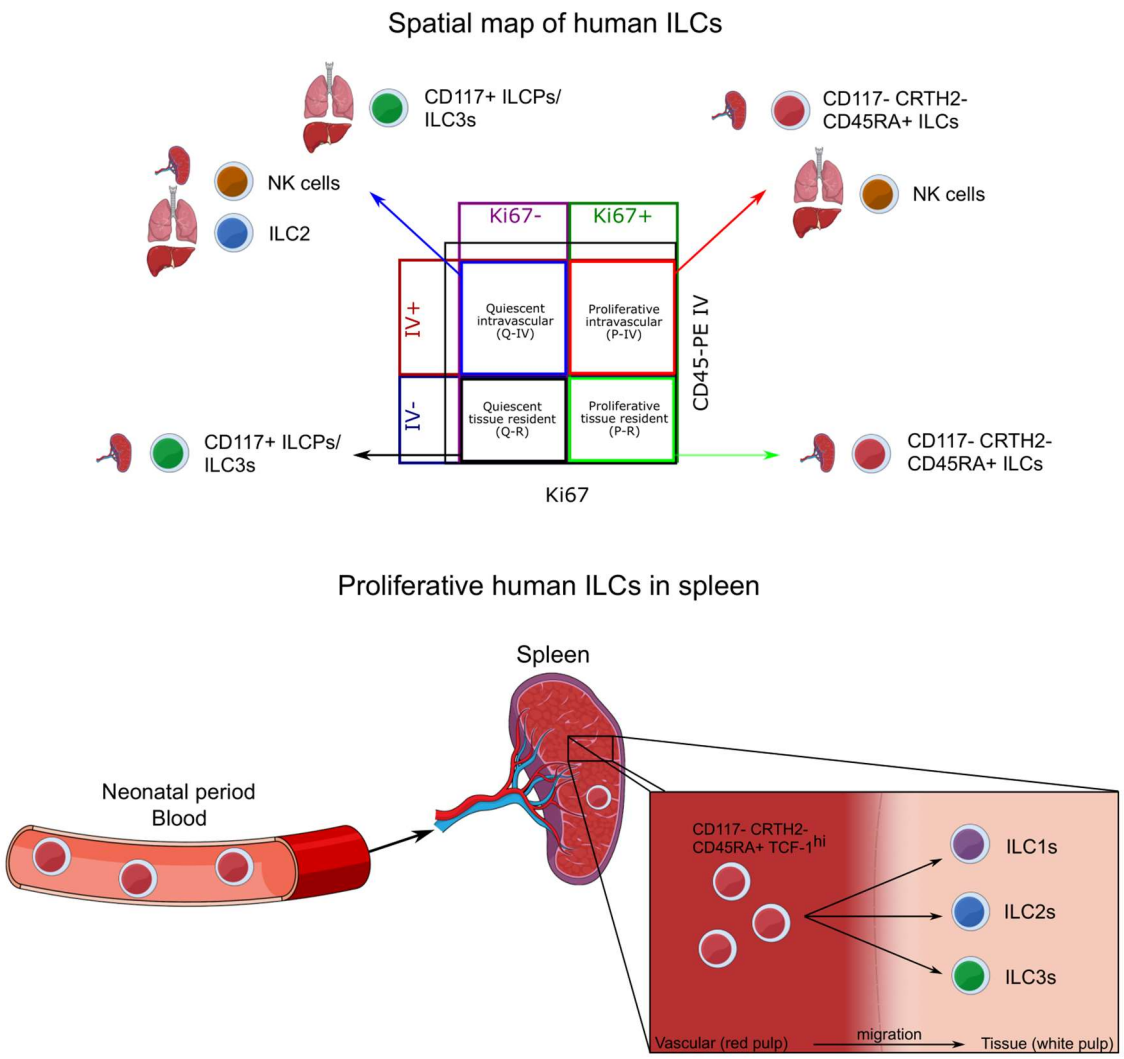


**Figure 8.** Graphical summary of Paper II

## 4.3 MOLECULAR SIGNATURES OF HUMAN ILCS AND NK CELLS IN THE VASCULAR AND TISSUE NICHES IN THE LUNG

From our previous two studies, we found that ILCs are distributed between the vascular and tissue compartments of organs and that ILC subsets have specific functions in relation to their localization. However, the mechanisms governing ILC migration, their spatial compartmentalization, and the impact of cues from the local microenvironment on human lung ILC identity remain unclear, especially in the human context.

### 4.3.1 Human ILCs are compartmentalized between the lung vasculature and tissue in MISTRG mice

Lung ILCs have been studied in both mice and humans. Mouse studies have been invaluable to our understanding of ILC biology, but there are several important differences from human ILCs[115]. One such difference is that ILC2s are the predominant ILC subset in the mouse lung[23,136] whereas ILC3s are the predominant subset in the human lung[18,20,137]. Moreover, human studies are more limited due to experimental limitations. For example, spatial information of cells isolated from a human tissue sample is often lost. Our humanized

MISTRG mouse model allowed us to study the migration and localization of human lung ILCs *in vivo*. The distribution of human ILC subsets in the MISTRG lung closely resembles that of ILCs in the human lung (Paper III, Fig. 1A-B). Another advantage of this model is that it allows us to use intravascular cell labelling (Paper III, Fig. 1C) to distinguish cells from the vascular and tissue compartments of the lung. This method has been used in our previous studies[110,124,125] and in Paper III we validated it further using the known tissue resident markers CD103 and CD69[138]. $CD103^+$ ILCs, which are known to localize among epithelial cells[86] were not labeled by the IV CD45-PE antibody (Paper III, Fig. 1E). $CD69^+$ ILCs were also predominantly found within the cells that were not labeled by the IV CD45-PE antibody. However, we also observed that the frequency of $CD69^+$ ILC2s and ILC3s was higher in the lung vasculature than in the peripheral blood. This observation suggests that these subsets already start expressing tissue residency markers when migrating from the local vasculature into the lung tissue. This pattern of gradual CD69 upregulation in ILC2s and ILC3s was not observed in ILC1s and NK cells, suggesting differences in migratory function between these two groups. ILC2s and ILC3s in the lung vasculature are likely marginated cells, whereas ILC1s and NK cells are likely recirculating. Overall, this result highlights the distinction of human ILCs residing in the different anatomical compartments (peripheral blood, lung vascular, extravascular lung tissue).

### 4.3.2 Human lung ILC subsets are distributed between vasculature and tissue

To investigate which signals shape the localization, migration, and tissue residency of human lung ILCs, we carried out single cell RNA-sequencing. IV $CD45\text{-}PE^+$ and IV $CD45\text{-}PE^-$ human ILCs and NK cells were sorted from lung cells isolated from 9-10 HSPC-engrafted MISTRG mice. We identified 8 different ILC clusters expressing characteristic markers of the main ILC subsets (Paper III, Fig. 2A-B). Cluster identity was confirmed by performing module score analysis using known core ILC signatures[20]. Individual clusters were mostly distributed in either the vascular or the tissue compartment, with each main ILC subset represented by multiple clusters that were either predominant in the vasculature or the lung tissue (Paper III, Fig 3A-B). For example, cluster 1 and 4 were both ILC2 clusters, but cluster 1 was predominantly in the lung tissue, whereas cluster 4 was more abundant in the lung vasculature. Similarly, cluster 2 and 5 were both ILC3s with cluster 2 being predominant in the lung tissue and cluster 5 being enriched in the lung vasculature. Different features were found in clusters of the same subset in different locations, for example tissue resident ILC2s (cluster 1) expressed ILC2 genes and the transcription factor *RORA* that is required for ILC2 development[139]. On the other hand, vascular ILC2s (cluster 4) expressed characteristic ILC2 transcription factors, mRNA for cell surface proteins (*PTGDR2* (encoding CRTH2), *KLRG1*), the cytokine *IL13* and genes involved in prostaglandin metabolism (*HPGD*, *HPGDS*). Vascular ILC3s (cluster 5) expressed *ZBTB16* encoding PLZF, a transcription factor required for ILC development from precursors[140]. Tissue-resident lung ILC3s (cluster 2) shared characteristics with LTi-like cells such as the expression of MHC class II genes, cytokines from the TNF family and genes encoding the surface receptors TNFRSF4 and TNFRSF18. We also identified a cluster of tissue ILC3s (cluster 0), a cluster corresponding to naïve ILCs (cluster 3) that were predominantly intravascular and expressed genes associated with $CD117^+$ circulating ILCPs[55,57], an intravascular ILC1 cluster (cluster 6) and a proliferating ILC cluster expressing *MKI67* (cluster

7).By performing module score analysis on the different ILC clusters using published human blood and tissue ILC signatures[20] (Paper III, Fig 3C-D), we found that the distribution of ILC clusters in the lung vascular and tissue compartment matched the vascular and tissue counterparts in humans. Here, we conclude that spatial niches imprint different transcriptional signatures on human lung ILCs.

### 4.3.3 Human lung CD56$^{dim}$ and CD56$^{bright}$ NK cells are distributed between vasculature and tissue

Our main focus in this study was the helper ILC subsets (ILC1s, ILC2s and ILC3s), but we included NK cells for a comparative analysis. Six different NK cell clusters were identified by single-cell RNA-sequencing in the lung of HSPC-engrafted MISTRG mice, corresponding to three CD56$^{dim}$ subsets, one CD56$^{bright}$ subset, and one transitional NK cell subset as well as a MKI67 expressing proliferative subset (Paper III, Fig. 4A-B). CD56$^{dim}$ subsets expressed cytolytic molecules and were distinguished from each other by the expression of MHC class II, chemokines, and cytokines such as *IFNG*. CD56$^{bright}$ NK cells (cluster 4) had lower expression of cytolytic genes and higher expression of cytokine and chemokines genes, such as *CSF2*, *LTB2, AREG, XCL1* and *XCL2*. Cluster identities were confirmed by module score analysis using signatures of CD56$^{dim}$ and CD56$^{bright}$ NK cells from the human lung as published in Dobra et al. 2020., This analysis showed that transitional NK cells (cluster 2) and the proliferative NK cell cluster (cluster 5) had intermediate scores in terms of their similarity to CD56$^{dim}$ and CD56$^{bright}$ NK cells (Paper III, Fig. 4C). Interestingly, all three CD56$^{dim}$ NK cell clusters (cluster 0, 1, 3) were found mostly in the lung vasculature, whereas the CD56$^{bright}$ cluster (cluster 4) and transitional NK cells (cluster 2) were found predominantly in the lung tissue (Paper III, Fig. 4D-E). Unlike lung ILCs, the two main subsets of lung NK cells (CD56$^{dim}$ and CD56$^{bright}$) seemed to be separated by their anatomical compartment. Furthermore, transitional or proliferative NK cells residing in the tissue could potentially act as precursors for CD56$^{dim}$ NK cells.

### 4.3.4 Molecular signature of tissue and vascular lung ILCs and NK cells

To gain further insight on how the local environment shapes human ILC and NK cell subsets, we identified genes that were differentially expressed in ILCs/NK cells from the lung vasculature versus ILCs/NK cells located in the lung tissue.

Genes regulating lymphocyte recirculation and migration such as *KLF2*, *SELL* (encoding CD62L), and S100 proteins were preferentially expressed by vascular ILC and NK cells subsets. Similarly, known tissue residency markers were upregulated by tissue ILCs and NK cells. These included *JAML*, *CXCR4*, *CD69* and genes regulating signaling downstream of G protein-coupled receptors (e.g.*RGS1* and *RGS16*) which are involved in cell migration (Paper III, Fig. 5A, 6A). This shows that some genes regulating migration and tissue residency are conserved in ILCs and NK cells.

Other shared genes expressed by extravascular ILCs, and NK cells pointed towards their functional role within the lung tissue. For example, ILCs and NK cells in lung tissue expressed genes involved in cellular adaptation to stress, such as immediate early genes (*JUNB*, *DUSP4*), transcription factors regulating immediate early genes (*CEBPB*, *NFKBIA*, *REL*) as well as heat

shock proteins (Paper III, Fig. 5B, 6B). Other processes included sensing of tissue-derived signals such as cytokines, Notch and WNT signals, as well as sensing of hypoxia and tissue acidosis (Paper III, Fig. 5C, 6C). Finally, tissue ILCs and NK cells expressed genes that regulate interactions with other immune cells, interaction e.g., with $CD4^+$ T cells through MHC class II genes (*HLA-DQA1*, *HLA-DRA*), with myeloid cells through chemokines (*XCL1, XCL2, CKLF*) and with B cells (*TNFSF13B*). Overall, our findings indicate that human ILCs and NK cells react to tissue specific cues when they migrate into the lung, which likely regulates their specific functional roles within the tissue compartment of the lung.

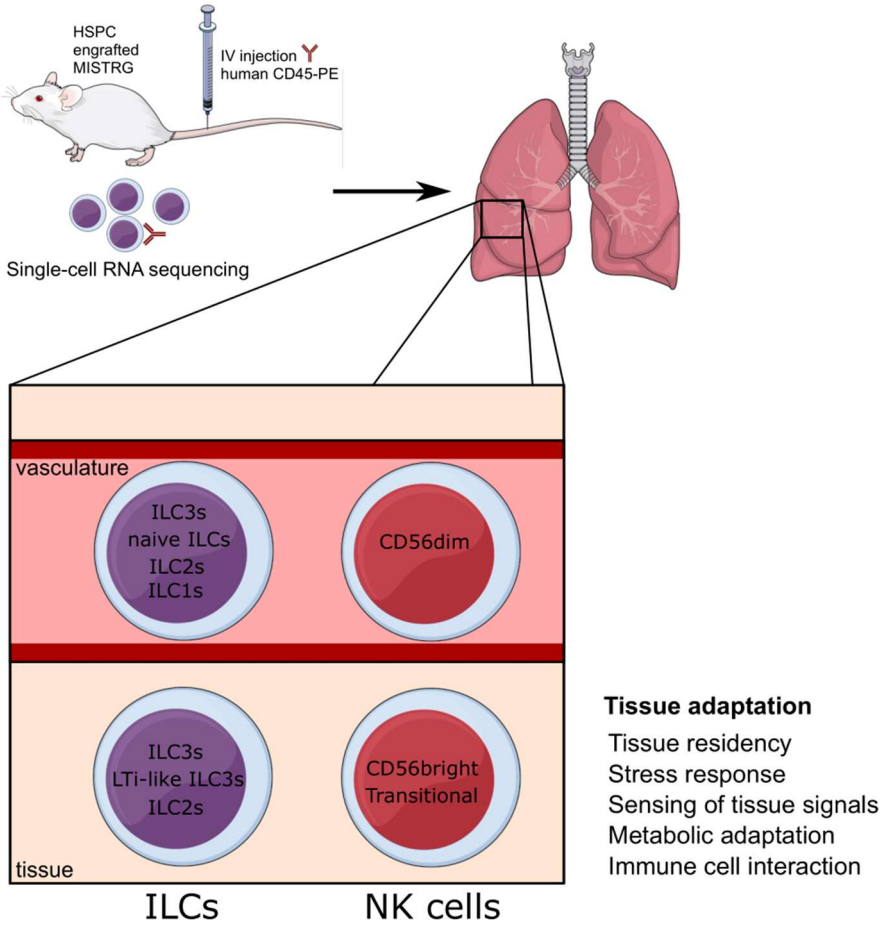


**Figure 9.** Graphical summary of Paper III

# 5 CONCLUSIONS

ILCs are a relatively new subset of immune cells, but substantial progress in the past decade has been made to further our understanding of their development and function. A large amount of our knowledge on ILCs is derived from mouse studies. However, due to differences between mice and humans, these findings are not always translatable and *in-vivo* knowledge about human ILCs is more limited. The studies with the MISTRG humanized model presented in this thesis contribute to the knowledge on the spatial distribution of human ILCs in relation to their ontogeny and function. Key findings in this thesis are:

- Human $CD5^+$ ILCs have been described, but of their properties have only been investigated *in vitro*. In Paper I we demonstrate *in vivo* that $CD5^+$ ILCs are a heterogenous population distinct from conventional ILCs. $CD5^+$ ILCs consisted of mature ILC1s and an immature ILC population that resides in the vasculature and has a distinct ontogeny. The specific localization of $CD5^+$ ILCs in the lung vasculature could point to their role as sentinels that patrol the blood vessels and migrate into the tissue in response to lung injury.
- In Paper II, we mapped the spatial distribution of human ILC subsets in four organs and identified sites of human ILC proliferation. We also discovered highly proliferative $CD117^-$ $CRTH2^-$ $CD45RA^+$ ILCs in the spleen that expressed the "stemness" transcription factor TCF-1. These proliferative ILCs were present in human umbilical cord blood and appeared early in MISTRG mice engrafted with human HSPCs. Our results link human ILC proliferation with their spatial compartmentalization. Furthemore, our findings suggest the spleen as a site of human ILC proliferation.
- In Paper III we used single-cell RNA-sequencing and intravascular cell labeling to determine *in vivo* the molecular features of human lung ILCs and NK cells in the vascular and tissue compartment. We discovered that human ILC subsets are heterogenous and present in both the lung vascular and tissue compartment of HSPC-engrafted MISTRG mice. In contrast, human $CD56^{dim}$ and $CD56^{bright}$ NK cells in the MSITRG lung were strictly divided between the two compartments. We also identified a conserved molecular profile between human ILCs and NK cells localized in the lung tissue.This finding shows how distinct anatomical niches in the lung shape the migration, heterogeneiety, and function of human ILCs and NK cells.

In conclusion, the vascular and tissue compartment of organs are different environmental niches that influence human ILC heterogeneity and regulate their function. ILCs in the intravascular compartment likely serve as sentinels or an immature reservoir, ready to be recruited into the tissue and replenish cells during inflammation. ILCs in the extravascular compartment respond to tissue signals, perform specialized functions, and communicate with other immune cells.

# 6 POINTS OF PERSPECTIVE

Our results in Paper I introduced the possible role of human $CD5^+$ ILCs as blood-borne sentinels. Further studies are needed to fully investigate their role in inflammation and disease. The MISTRG humanized mouse model offers a unique opportunity to study this *in vivo* using infection models. In-depth characterization of the adhesion and chemokine receptors on the human $CD5^+$ ILC population would reveal mechanisms governing their migration into specific tissues, which could open up the possibility to target them for the development of novel treatments. Furthermore, it would be interesting to further investigate the functional properties of CD5 in this human ILC population. Our results indicate that CD5 may have an inhibitory function to keep $CD5^+$ ILCs in an immature state. Bulk culture *in vitro* results suggests that $CD5^+$ ILCs could contain an ILCP population. However, to fully confirm this, single-cell ILC cloning *in vitro* and adoptive transfer *in vivo* need to be performed. Finally, as $CD5^+$ ILCs and ILC1s in general have some T cell characteristics, such as intracellular CD3 expression, it will be interesting to further elucidate their lineage identity in relation to T cells. Recent studies have shown that ILC1s express *TCR* transcripts, which raises the possibility that failed *TCR* rearrangement could diverge T lymphocyte development into the ILC lineage.

In Paper II, we identified a population of $CD117^-$ $CRTH2^-$ $CD45RA^+$ $TCF\text{-}1^+$ ILCs in the spleen that were highly proliferative. It is unclear whether this population is an ILCP or immature "naïve" ILCs. Therefore, further characterization of this subset by *in-vivo* adoptive transfer and by single-cell cloning experiments *in vitro* is required. Furthermore, our results indicate that the spleen could be a niche for human ILC proliferation. To test this notion, splenectomized MISTRG mice could be employed. Characterization of signals and receptors regulating the localization of this population in the spleen would further our understanding of the molecular mechanisms underlying their migration. As a proliferative population, it would also be interesting to see how the $CD117^-$ $CRTH2^-$ $CD45RA^+$ ILCs respond to infection, inflammation, and tissue injury, whether they migrate from the spleen into inflamed tissues via the circulation and/or whether they contribute to host defense.

In paper III, we found that, while heterogeneous, human ILCs and NK cells share a molecular signature in relation to their vascular and tissue distribution in the lungs. Further research is needed to validate the differentially expressed genes at the protein level. For example, surface protein expression and cytokine production could be validated by flow cytometry. Functional validation of migratory receptors could be performed *in vivo* by blocking the receptors in HSPC-engrafted MISTRG mice using antibodies. As a next step, models for lung infection and injury could be employed to further elucidate their role in these contexts. Further defining the ILC clusters identified in this study using surface markers and depleting the subset using neutralizing antibodies is another possibility. With sufficient knowledge, we could modulate the trafficking and migration of human ILC and NK cells subsets to influence the outcome of human disease.

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