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INNATE LYMPHOID CELL DIFFERENTIATION AND FUNCTIONS IN INTESTINAL HOMEOSTASIS AND DISEASE

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Cover illustration: 'Connecting dots'. Abstract one-line figure of the intestinal epithelium (top) and lamina propria (bottom). The red color indicates intestinal disease/inflammation. The orange circles depict the ILCs within the lamina propria. Designed by Efthymia Kokkinou.

INNATE LYMPHOID CELL DIFFERENTIATION AND FUNCTIONS IN INTESTINAL HOMEOSTASIS AND DISEASE

THESIS FOR DOCTORAL DEGREE (Ph.D)

By

Efthymia Kokkinou

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To my mother,

if you were born with the weakness to fall

you were born with the strength to rise

-rupi kaur

POPULAR SCIENCE SUMMARY OF THE THESIS

Ever wondered how many cells are present within our body? The answer is the astonishing number of 37 trillion cells! Innate lymphoid cells (ILCs in short) are a fairly newly discovered immune cell type that are equipped with specialized functions: to fight against pathogens, tumors, bacteria, even help in organogenesis! They are quite rare in our body, but nevertheless carry out important functions. These cells prefer to reside within tissues and to swim around proteins that can make them active and produce even more proteins. Additionally, ILCs, like ice-creams, come in different flavors, so called, ILC1, ILC2 and ILC3. In certain aspects ILCs are twins with T cells, which is another immune cell type, because they indeed look very much alike.

Due to their functions and preferential localization, it is interesting to study them in diseases where tissues are affected such as Inflammatory Bowel Disease (IBD) and colorectal cancer (CRC). For example, in IBD, the intestine gets inflamed, causing pain, fatigue, diarrhea and generally low quality of life in the patients that suffer from it. It is therefore interesting to understand the 'how?when? and whys?' of the disease pathogeneses by learning more and new aspects of this immune cell type.

In this thesis, we characterized the functional characteristics and potentials of a 'dormant' state of ILCs. We found that these 'dormant' cells can under certain conditions become awake and generate other ILCs. The cells had this ability in tissues obtained from patients with IBD and, therefore, this opens new avenues for tackling the disease.

We also explored this cell type in pediatric patients with IBD. There we showed that the ILCs of the patients with IBD are not similar in frequencies to those patients with a healthy gut. Moreover, we described which programs ILCs and T cells have in common, and could therefore, justify why they are considered twins!

Lastly, we described the functional characteristics of an interesting subpopulation of ILCs; the one that expresses a receptor (HLA-DR) and makes them communicate with their twins, the T cells!

All in all, our findings could be this next piece in the puzzle that brings forward new information and potentially help, in the long run, the identification of novel therapies for these diseases.

ΠΕΡΙΛΗΨΗ ΔΙΑΤΡΙΒΗΣ ΜΕ ΑΠΛΑ ΛΟΓΙΑ

Αναρωτηθήκατε ποτέ πόσα κύτταρα υπάρχουν στο σώμα μας; Η απάντηση είναι ο εξωπραγματικός αριθμός των 37 τρισεκατομμυρίων κυττάρων! Τα έμφυτα λεμφοειδή κύτταρα (ILCs εν συντομία) είναι ένας τύπος κυττάρων του ανοσοποιητικού που εξειδικεύονται στο να καταπολεμούν παθογόνους οργανισμούς, όγκους/καρκινικά κύτταρα, βακτήρια, ακόμη και να βοηθούν στην οργανογένεση! Είναι αρκετά σπάνια στον οργανισμό μας, παρόλα αυτά επιτελούν σημαντικές λειτουργίες ;! Αυτά τα κύτταρα προτιμούν να κατοικούν μέσα σε ιστούς και να κολυμπούν γύρω από πρωτεΐνες που μπορούν να τα καταστήσουν ξύπνια, ενεργά και για να παράγουν ακόμη περισσότερες πρωτεΐνες. Επιπλέον, τα ILCs, όπως τα παγωτά, βγαίνουν σε διαφορετικές γεύσεις, τα λεγόμενα ILC1, ILC2 και ILC3. Από ορισμένες απόψεις τα ILCs είναι δίδυμα με τα Τ-λεμφοκύτταρα, που είναι ένας άλλος τύπος ανοσοκυττάρων επειδή, πράγματι, μοιάζουν πολύ!

Λόγω των λειτουργιών τους και του προτιμώμενου εντοπισμού τους, είναι ενδιαφέρον να μελετηθούν σε ασθένειες όπου προσβάλλονται ιστοί όπως είναι οι ιδιοπαθείς φλεγμονώδεις νόσους του εντέρου (ΙΦΝΕ, IBD) και ο καρκίνος του παχέος εντέρου (CRC). Για παράδειγμα, στη φλεγμονώδη νόσο του εντέρου, το έντερο φλεγμονώνεται, προκαλώντας πόνο, κόπωση, διάρροια και γενικά χαμηλή ποιότητα ζωής στους ασθενείς που υποφέρουν από αυτήν. Είναι λοιπόν ενδιαφέρον να κατανοήσουμε τα «Πώς; Πότε; και Γιατί;» της παθογένειας της νόσου μαθαίνοντας περισσότερες και νέες πτυχές αυτού του τύπου ανοσοκυττάρων.

Σε αυτή τη διατριβή, χαρακτηρίσαμε τα λειτουργικά χαρακτηριστικά και τις δυνατότητες μιας «αδρανούς» κατάστασης των ILCs. Βρήκαμε ότι αυτά τα «αδρανή» κύτταρα μπορούν υπό ορισμένες συνθήκες να αφυπνιστούν και να δημιουργήσουν άλλα ILCs. Τα κύτταρα αυτά είχαν την ικανότητα αυτή και σε ιστούς που ελήφθησαν από ασθενείς με ΙΦΝΕ και επομένως αυτό ανοίγει νέους δρόμους για την αντιμετώπιση της νόσου.

Εξερευνήσαμε επίσης αυτόν τον τύπο κυττάρων σε παιδιατρικούς ασθενείς με ΙΦΝΕ. Εκεί δείξαμε ότι τα ILCs των ασθενών με ΙΦΝΕ βρίσκονται σε δυσανάλογες συχνότητες με εκείνους τους ασθενείς με υγιές έντερο. Επιπλέον, περιγράψαμε ποια προγράμματα λειτουργίας κυττάρων είναι κοινά μεταξύ των ILC και των Τ λεμφοκυττάρων, και που επομένως θα μπορούσαν να δικαιολογήσουν γιατί οι άνθρωποι τα αποκαλούν δίδυμα!

Τέλος, περιγράψαμε τα λειτουργικά χαρακτηριστικά ενός ενδιαφέροντος υποπληθυσμού των ILCs: αυτού που εκφράζει έναν υποδοχέα (HLA-DR) και τους κάνει να επικοινωνούν με τα δίδυμα τους, τα Τ λεμφοκύτταρα!

Συνολικά, τα ευρήματά μας θα μπορούσαν να είναι αυτό το επόμενο κομμάτι του παζλ που φέρνει νέες πληροφορίες και ενδεχομένως μπορεί να βοηθήσει, μακροπρόθεσμα, τον εντοπισμό καινοτόμων θεραπειών για αυτές τις ασθένειες.

ABSTRACT

The gastrointestinal (GI) tract, which includes the small and the large intestine, is considered the largest immunological organ that plays a pivotal role in food digestion, nutrient absorption and fuel generation. Inflammatory Bowel Disease (IBD), which is broadly divided into Crohn's disease (CD) and Ulcerative Colitis (UC), are multifactorial chronic intestinal inflammatory conditions that affect both pediatric and adult patients. Patients with IBD have a significantly increased risk of developing colorectal cancer (CRC). The immune system is undoubtably a major factor in disease pathogenesis and understanding what goes awry to cause disease is of great importance. This thesis focuses on a particular immune cell type called innate lymphoid cells (ILCs), and follows our journey to understand their role in the intestine of pediatric and adult patients with IBD as well as in CRC.

Since the discovery of ILCs is fairly recent, a lot remains unknown regarding their phenotype and function at steady and disease state, particularly in humans. In this thesis we performed immunophenotypic, functional, transcriptional as well as epigenetic assays to understand many aspects of their biology, differentiation and interactions with their adaptive counterparts, T cells.

In **Paper I**, we described the presence of $CD45RA^+$ ILCs with naïve features in the tonsil that are transcriptionally, epigenetically and functionally distinct from the differentiated ILC subsets. We demonstrated that ($CD45RA^+$)$CD62L^-$ ILCs were accumulated in the inflamed gut of adult patients with IBD and compared to their tonsil counterpart, these cells showed preferential differentiation towards IL22-producing ILC3s. In **Paper II**, we determined the landscape of innate and adaptive lymphocytes in pediatric IBD (pIBD) through single-cell RNA sequencing. First, we demonstrated that ILCs are altered in pIBD, and in line with Paper I, we show that increased frequency of $CD62L^-$ ILCs is also a feature in pediatric IBD patients. Also, we were able to uncover shared and unique transcriptional signatures between ILCs and T cells and identify with a neighbor-based computational method the most and least inflamed cells in our dataset. In **Paper III**, we explored the antigen-presenting properties of circulating and tissue-resident intestinal ILCs. Specifically, we were able to demonstrate that human circulating $HLADR^+$ ILCs were able to internalize, process and present antigen to memory $CD4^+$ T cells, and that this process is regulated by IL-1β through NF-κB signaling while it is suppressed by TGF-β. Additionally, via confocal microscopy we found that intestinal $HLADR^+$ ILCs were located in close proximity to T cells, raising the possibility that the antigen presentation by ILCs could occur *in vivo.*

Overall, the research work included in this thesis contributes to advancing our understanding of ILCs in intestinal homeostasis and disease. The characterization of novel ILC states and functions in complex intestinal diseases such as IBD and CRC could pave the way for unraveling mechanisms that drive these diseases and potentially facilitate the development of more effective therapies for those patients.

LIST OF SCIENTIFIC PAPERS

I. **Efthymia Kokkinou**, Ram Vinay Pandey, Luca Mazzurana, Irene Gutierrez-Perez, Christopher Andrew Tibbitt, Whitney Weigel, Tea Soini, Anna Carrasco, Anna Rao, Maho Nagasawa, Suzanne M Bal, Mattias Jangard, Danielle Friberg, Ulrik Lindforss, Caroline Nordenvall, Malin Ljunggren, Staffan Haapaniemi, Åsa V Keita, Johan Söderholm, Charlotte Hedin, Hergen Spits, Yenan T. Bryceson, Jenny Mjösberg. $CD45RA^{+}CD62L^{-}$ ILCs in human tissues represent a quiescent local reservoir for the generation of differentiated ILCs. *Science Immunology*. 2022 Apr 15;7(70).

II. **Efthymia Kokkinou**, Tea Soini*, Ram Vinay Pandey*, Aline van Acker*, Jakob Theorell, Paulo Czarnewski, Egle Kvedaraite, Niels Vandamme, Magda Lourda, Chiara Sorini, Whitney Weigel, Anna Carrasco, Christopher Andrew Tibbitt, Heinrich Schlums, Ulrik Lindforss, Caroline Nordenvall, Malin Ljunggren, Maja Ideström, Mattias Svensson, Jan-Inge Henter, Eduardo J. Villablanca, Yenan T. Bryceson, Helena Rolandsdotter, Jenny Mjösberg. The single-cell transcriptional landscape of innate and adaptive lymphocytes in pediatric-onset colitis. *Manuscript submitted.*

III. Anna Rao, Otto Strauss*, **Efthymia Kokkinou***, Mélanie Bruchard, Kumar P. Tripathi, Heinrich Schlums, Anna Carrasco-Garcia, Luca Mazzurana, Viktoria Konya, Eduardo J. Villablanca, Niklas K Björkström, Ulrik Lindforss, Hergen Spits, Jenny Mjösberg. Cytokines regulate the antigen-presenting characteristics of human circulating and tissue-resident intestinal ILCs. *Nature Communications.* 2020 Apr 27;11(1):2049.

*equal contribution

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

(Chronological order)

I. Hans-Gustaf Ljunggren, Eivind Heggernes Ask, Martin Cornillet, Benedikt Strunz, Puran Chen, Jagadeeswara Rao Muvva, Mira Akber, Marcus Buggert, Benedict J. Chambers, Majda Dzidic, Iva Filipovic, Marina Garcia, Jean-Baptiste Gorin, Sara Gredmark-Russ, Laura Hertwig, Jonas Klingström, **Efthymia Kokkinou**, Egle Kvedaraite, Magdalini Lourda, Jenny Mjösberg, Christopher Maucourant, Anna Norrby-Teglund, Tiphaine Parrot, André Perez-Potti, Andrea Ponzetta, Olga Rivera-Ballesteros, Olav Rooyackers, Johan K. Sandberg, John Tyler Sandberg, Takuya Sekine, Mattias Svensson, Renata Varnaite, Karolinska KI/K COVID-19 Study Group, Lars I. Eriksson, Soo Aleman, Karl-Johan Malmberg, Kristoffer Strålin, and Niklas K. Björkström. The Karolinska KI/K COVID-19 Immune Atlas: An open resource for immunological research and educational purposes. *Scandinavian Journal of Immunology.* 2022 Jun 2;e13195.

II. Martin Cornillet, Benedikt Strunz, Olav Rooyackers, Andrea Ponzetta, Puran Chen, Jagadeeswara Rao Muvva, Mira Akber, Marcus Buggert, Benedict J Chambers, Majda Dzidic, Iva Filipovic, Jean-Baptiste Gorin, Sara Gredmark-Russ, Laura Hertwig, Jonas Klingström, **Efthymia Kokkinou**, Egle Kvedaraite, Magda Lourda, Jenny Mjösberg, Christopher Maucourant, Anna Norrby-Teglund, Tiphaine Parrot, André Perez-Potti, Olga Rivera-Ballesteros, Johan K Sandberg, John Tyler Sandberg, Takuya Sekine, Mattias Svensson, Renata Varnaite, Karolinska KI/K COVID-19 Study Group, Lars I Eriksson, Soo Aleman, Kristoffer Strålin, Hans-Gustaf Ljunggren, Niklas K Björkström. COVID-19-specific metabolic imprint yields insight into multiorgan system perturbations. *European Journal of Immunology*. 2022 Mar;52(3):503-510.

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VI. **Efthymia Kokkinou**, Jenny Mjösberg. Tummy time for ILC2. *Immunity.* 2020 Apr 14;52(4):573-575.

VII. Luca Mazzurana, Marianne Forkel, Anna Rao, Aline Van Acker, **Efthymia Kokkinou**, Tamaki Ichiya, Sven Almer, Charlotte Höög, Danielle Friberg, Jenny Mjösberg. Suppression of Aiolos and Ikaros expression by lenalidomide reduces human ILC3-ILC1/NK cell transdifferentiation. *European Journal of Immunology.* 2019 Sep;49(9):1344-1355.

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LIST OF ABBREVIATIONS

αLP	α-lymphoid precursor
AHR	Aryl hydrocarbon receptor
AMPs	Antimicrobial peptides
APC	Antigen presenting cell
AREG	Amphiregulin
ATAC-seq	Assay for transposase accessible chromatin using sequencing
BM	Bone marrow
CD	Crohn’s Disease
CHILP	Common ‘helper’ ILC precursor
CILP	Common ILC progenitor
CLP	Common lymphoid precursor
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disorder
CRSwNP	Chronic rhinosinusitis with nasal polyps
CRTH2	Chemoattractant Receptor for Th2 cells
DC	Dendritic cell
EIPL	Early ILC progenitor
ETP1	Early tonsillar progenitor 1
ETP2	Early tonsillar progenitor 2
FACS	Fluorescence activating cell sorting
HSC	Hematopoietic stem cells
IBD	Inflammatory bowel disease
ieILC1	Intraepithelial ILC1
ILC	Innate lymphoid cell
iILC2	Inflammatory ILC2
ILC3P	ILC3-restricted precursor
ILCP	Innate lymphoid cell precursor
KIRs	Killer-like immunoglobulin receptors

LT	Lymphotoxin
LTi	Lymphoid tissue inducer cells
MCMV	Murine cytomegalovirus
MHC	Major Histocompatibility complex
MNP	Mononuclear phagocytes
MSCs	Mesenchymal stromal cells
MΦs	Macrophages
MLP	Multi-lymphoid precursor
mRNA	Messenger RNA
nILC2	Natural ILC2
NCR	Natural cytotoxicity receptor
NK cell	Natural killer cell
NKP	Natural killer cell precursor
NmU	Neuromedin U
NRP1	Neuropilin 1
OSA	Obstructive sleep apnea
PB	Peripheral blood
pIBD	Pediatric IBD
RA	Retinoic acid
scRNA-seq	Single-cell RNA sequencing
SLOs	Secondary lymphoid organs
TCR	T cell receptor
TGF-β	Tumor growth factor-β
TF	Transcription factor
TL1A	TNF-like ligand 1A
Tn5	Transposase 5
TNF-α	Tumor necrosis factor-α
trNK	Tissue resident NK cell
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis

1 INTRODUCTION

1.1 INNATE LYMPHOID CELLS

The discovery of Natural Killer (NK) cells in 1975 as a non-T, non-B lymphocyte population with 'natural' cytotoxicity activity against tumour cells paved the way for the subsequent identification of Innate Lymphoid Cells (ILCs) in 2008-2009 *(1)*. ILCs show similarities with T helper (Th) cell subsets, but unlike T cells, they lack rearranged antigen-specific receptors and, therefore, are defined by their cytokine production *(2)*. ILCs have been categorized into five distinct subsets based on their cytokine and transcription factor expression. In addition to NK cells and lymphoid tissue inducer cells (LTi), these are the group 1 ILCs (ILC1s), group 2 ILCs (ILC2s) and the group 3 ILCs (ILC3s), which in humans are further sub-divided into $NKp44^{+}$ and $NKp44^{-}$ ILC3s (Figure 1).

Although ILCs can be found in the circulation, a quintessential feature is that they reside within tissues where they can exert protective or inflammatory responses *(3)*. Circulating and tissue-resident ILCs have profound differences in their phenotype and function. While circulating ILCs are considered more naïve/immature, tissue-resident ILCs are generally more prone to activation due to their constant stimulation by local environmental cues. Perhaps as a reflection of tissue residency and the impact of the microenvironment on ILC function, recent studies have revealed profound tissue-specific transcriptional signatures and heterogeneity of human *(4–6)* and mouse ILCs *(7)*, suggesting that they may be more diverse than originally proposed. In the present thesis, the main focus is on tissue-residing intestinal ILC subsets and the impact of the tissue microenvironment on their differentiation and functions as well as the transcriptional differences, similarities and functional interactions with T cells.

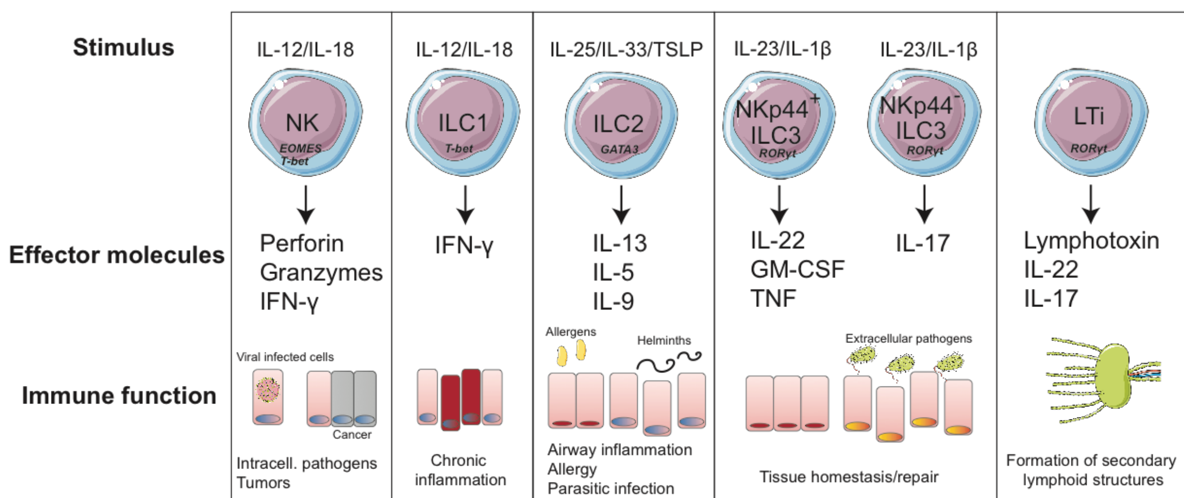

Figure 1. Graphical illustration of the human ILC subsets. Icons derive from Servier Medical Art.

1.1.1 ILC1 and NK cells

The group 1 ILCs is a term that used to include both the conventional non-cytotoxic ILC1s and NK cells, due to their similarities. While both subsets have the ability to secrete IFN-γ *(8)*, ILC1s depend on the transcription factor T-bet (encoded by *TBX21*) and IL-7 for their development, whereas NK cells depend on Eomesodermin (encoded by *EOMES*) and IL-15 and additionally have cytotoxic capacity *(9)*.

Multiple studies over the years have argued the case that ILC1s are a distinct lineage especially in humans *(10)*. Some of the reasons include 1) the lack of lineage defining markers compared to other ILC subsets, 2) the expression of transcripts related to the T cell lineage (CD3 chains, CD5, CD4, CD8) and non-productive TCR rearrangements, thus describing them as 'failed T cells' or lineage contaminants *(8, 11)*, 3) the failure to be detected across human organs in one study *(12)*, 4) the reported plasticity to ILC1-like cells from other ILC subsets and 5) the indefinite overlap with NK cells, including indistinguishability after *in vitro* cultures with $CD34^+$ hematopoietic progenitors *(13)*. The latter supports the idea that within tissue sites and under the influence of environmental signals, the phenotypes and functions of ILC1s can often be intertwined with those of NK cells making them overall challenging to study.

In contrast to NK cells, ILC1s exert low cytotoxic activity. In mice, ILC1s have demonstrated protection against intracellular infections such as *Toxoplasma gondii (14)* and *Salmonella enterica (15)* as well as, recently, in acute liver injury *(16)*. In fact, *Toxoplasma gondii* infection has been shown to drive the differentiation of NK cells to ILC1-like cells *(17)*.

In humans, ILC1s include intraepithelial ILC1s (ieILC1s) and conventional $CD127^+$ ILC1s. Intraepithelial ILC1s naturally reside within epithelial microenvironments and are functionally similar to NK cells *(12)*. Characteristic features of this subsets are the TGF-β imprinting, the expression of the $\alpha_e\beta_7$ integrin (CD103), T-bet and Eomes and the lack of CD127 expression *(18)*. They are also remarkably similar to resident memory $CD4^+$ and $CD8^+$ T cells exemplified by common expression of CD49a and Hobit, which are both linked to tissue residency program in T cells *(19)*. Interestingly, ieILC1-like cells with antitumor activity were recently implicated in head and neck cancer where they transdifferentiated from conventional NK cells *(20)*. Additionally, they have been found to expand in CD patients and contribute to inflammation in a mouse model of colitis *(18)*. The $CD127^+$ ILC1s are typically defined as lineage $(Lin)^-$ $CD127^+CD161^+CD117^-CRTH2^-$ and are present in tonsil, blood and gut at varying frequencies depending on the tissue and disease context. Although ILC1s are generally defined by absence of surface marker expression, peripheral blood (PB) IFN-γ-producing-ILC1s were found to express the Th1-associated chemokine receptor CXCR3, thus making it a potentially useful marker to better define ILC1s in blood *(11)*.

NK cells were discovered at the Karolinska Institutet in the mid-70's *(21)* and a decade later the "missing-self" concept was introduced where murine NK cells demonstrated the ability to kill a lymphoma cell line that lacked major histocompatibility complex-I (MHC-I) expression *(22)*. In humans, NK cells have been mostly characterized in blood where they are divided into

two major subsets, $CD56^{bright}$ and $CD56^{dim}$, and are inherently equipped with the capacity to kill foreign, infected and malignant cells *(23)*. The $CD56^{bright}$ additionally express NKG2A and low levels of CD127 and are considered immature and poorly cytotoxic *(24)*. Conversely, $CD56^{dim}$ represents the most abundant NK cell subset in PB and can express Killer-like Immunoglobulin Receptors (KIRs) and display high cytotoxic potential, including release of granzymes and perforin. It is now increasingly appreciated that NK cells have a broader diversity than originally thought, and this diversity takes into account factors such as surface, intracellular or signaling molecules, antigen exposure and the tissue microenvironment *(23)*. A detailed characterization of human NK cells by Dogra *et al*. identified tissue-specific signatures of NK cells in blood, lymphoid and non-lymphoid organs *(25)*. While blood, bone marrow, spleen and lungs contain mature and terminally differentiated NK cells, lymph nodes and intestines are dominated by precursor-like and immature NK cells *(25)*. Tissue-resident NK (trNK) cells with unique transcriptomes have been described in human lung *(26)*, while other studies demonstrated that $Eomes^{+}$ NK cells make up the majority of ILCs in human fetal intestine *(27)*.

Another feature of NK cells is immunological memory. Classically, memory has been described in antigen-specific immune cells, like T cells and B cells. However, it is now appreciated that memory could be also formed in non-antigen-specific cells, such as macrophages (MΦs) *(28)*, as well as epithelial stem cells *(29)*, and this feature is collectively termed as 'trained immunity'. The first report that described memory in NK cells was in the context of murine cytomegalovirus infection (MCMV) *(30)*. Since then, multiple studies have attempted to better understand the role and function of the so-called adaptive NK cells *(31)*.

1.1.2 ILC2

In humans ILC2s are defined by the expression of the surface molecules Chemoattractant Receptor for Th2 cells (CRTH2) and CD161 and are dependent on the transcription factor GATA3 *(32)*. They typically respond to epithelial- and myeloid-derived cytokines like IL-25, IL-33 and the thymic stromal lymphopoietin (TSLP) *(33)*. In response to those cytokines, they can produce type 2 cytokines such as IL-4, IL-13 and IL-5 which are implicated in respiratory disorders in humans *(34)*, as well as parasite expulsion and tissue repair in mice *(35)*. ILC2s can also be further amplified by leukotrienes, prostaglandins and neuropeptides *(36)*.

Although they were initially considered a rather homogenous population as compared to ILC1s and ILC3s, the diversity of human ILC2s is much broader than originally appreciated *(33)*, and it is affected by the tissue location which imprints their phenotype and function *(2)*. In mice, two subgroups of ILC2s have been described: the natural ILC2s (nILC2) which are tissue-resident and the inflammatory ILC2s (iILC2) that can migrate and appear upon exposure to inflammatory cytokine *(37)*.

In humans, circulating ILC2s is a heterogenous pool that contains KLRG1^{+} progenitor-like cells, that can give rise to mature CRTH2^{+} ILC2s *in vitro (38)*. However, CRTH2 is not a definite marker for 'maturity' as CD5^{+}CRTH2^{+} ILC2s from cord blood and postnatal thymus were unable to produce type 2 cytokines *(39)*. Additionally, CD117 discriminates two functionally distinct subsets in PB, with CD117^{+} ILC2s being the most 'plastic' form of ILC2 and CD117^{-} ILC2s forming a more committed ILC2 phenotype *(40)*. In the intestine ILC2s represent a dominant population in the mouse *(41)* and fetal gut *(32)*, but human adult gut is largely devoid of ILC2s *(12, 32)*. However, in the human lung, ILC2-like cells that lack expression of CRTH2 have been described *(4)*. This raises the possibility that human intestinal ILC2s might have a more unconventional profile than the one that classically defines them. It is, therefore, important to unravel factors beyond the already known, that could potentially orchestrate the presence of unconventional ILC2-like cells in the gut microenvironment.

Like NK cells, ILC2 have also displayed signs of immunological memory. This was supported by a study of a mouse model of allergic lung inflammation, where a subset of ILC2s could respond faster upon re-challenge *(42)*. In T cells, while CD45RA marks the naïve form of these cells, CD45RO is exclusively expressed by activated and memory T cells. In that regard, a recent study described conversion of CD45RA^{+} (resting ILC2s) to CD45RO^{+} ILC2s and these CD45RO^{+} ILC2s potentially represent the human equivalent of the mouse iILC2s *(43)*. However, whether this CD45RA-to-CD45RO switching is associated with ILC2 memory remains to be evaluated.

1.1.3 ILC3 and LTi

ILC3 and LTi are usually grouped together due to their mutual dependence on the ILC3-lineage defining transcription factor RORγt *(44)*. However, in mice, LTi derive from a distinct developmental trajectory than the helper ILC subsets *(45)*.

In humans ILC3s are defined as Lin^{-}CD127^{+}CD117^{+}CRTH2^{-} ILC3s that can either express or lack expression of the natural cytotoxicity receptor (NCR) NKp44 depending on the tissue they are present in. ILC3s are the dominant population in the intestinal tract, where they have been shown to mediate host defense against intestinal bacterial e.g, *Citrobacter rodentium (46), Clostridium difficile (47)* and fungal pathogens e.g *Candida ablicans (48)*, primarily due to their ability to secrete IL-22. It was recently reported that ILC3s can provide long term adaptation (so called 'trained' immunity) and can persist for months after exposure to the enterobacterial pathogen *Citrobacter rodentium (49)*, signs indicative of immunological memory in ILC3s.

ScRNA-seq studies have revealed that ILC3 is a rather heterogenous population in human mucosal tissues *(6)* and mouse intestine *(7)*. The human intestine is largely dominated by NKp44^{+} ILC3s *(50)* that produce IL-22 *(51)*, which is a key cytokine for maintaining the integrity of intestinal epithelial cells in mice *(52)* and human *in vitro* cultures *(53)*. NKp44^{-}

ILCs are described as being more 'resting' cells, due to their incapability to produce cytokines in the absence of activating signals, although are more prone to produce IL-17A/F than the NKp44^{+} counterparts *(51)*. In **Paper I**, we further explore the heterogeneity of the NKp44^{-} ILC pool as well as their involvement in Inflammatory Bowel Disease (IBD). Another transcriptionally distinct ILC3 subset expresses major histocompatibility complex II (MHCII) molecules (HLA-DR in humans) and is able to regulate T cell responses. More details on this subset are discussed in section 1.4 and were further explored in **Paper III**.

LTi cells, first reported in 1997 *(54)*, are essential for the formation of secondary lymphoid organs (lymph nodes, Peyer's patches) during fetal life *(55)*, through interactions with mesenchymal stromal cells (MSCs) and lymphotoxin (LT)-α1β2 production *(56)*. Although LTi in humans are less well-characterized compared to mouse, neuropilin-1 (NRP1) is a marker present on both mouse and human LTi-like cells *(57)*. NRP1^{+} ILC3 are described in the lungs of chronic obstructive pulmonary disorder (COPD) patients where they can form ectopic lymphoid aggerates through induction of ICAM-1 and VCAM-1 on MSCs *(57)*.

1.2 DEVELOPMENTAL PATHWAYS OF HUMAN INNATE LYMPHOID CELLS

Most of the existing knowledge concerning the development of innate lymphoid cells derives from mouse studies where *in vivo* experimentation is possible. However, a number of studies have made considerable efforts to delineate human ILC development, which is often paralleled to the developmental trajectories observed in mice. Fetal liver and bone marrow (BM) have been suggested as the main sites for generation of ILCs that are subsequently found in mucosal adult tissues *(58)*, while other studies in mice have identified progenitors in the fetal intestine as well *(59)*. In humans, $CD34^{+/-}$ ILC precursors have been identified in various sites including BM, fetal liver, lung, tonsils, blood and cord blood.

Lineage-committed ILCs derive from hematopoietic stem cells (HSC) that further differentiate to precursors biased towards development of lymphoid cells, commonly referred to as common lymphoid progenitors (CLP) *(60)* or multi-lymphoid precursors (MLP) *(61)*. Minimally these cells are defined as $Lin^{-}CD34^{+}CD45RA^{+}CD10^{+}$, but cells within this definition have also been shown to express e.g. CD117, CD127 and CD7 at varying frequencies, and are likely to contain cells with both multipotent and more restricted developmental potential *(61–63)*.

In mice, differentiation of ILCs from the CLP is orchestrated by several transcription factors that contribute differently at different stages of ILC development *(64)*. Fate mapping experiments have further helped in the identification of specialized ILC precursors such as the α-lymphoid precursors (αLP) *(65)*, the early ILC progenitor (EILP) *(66)* and the common 'helper' ILC precursor (CHILP) *(14, 67)* (Figure 2). Although αLP and EILP have very similar lineage potential, αLP is characterized by *Nfil3* and CD127 expression while EILP is negative for CD127 and expresses *Tcf7 (68)*. The CHILP, which is characterized by *Id2 (14)* and *Plzf* expression *(65)*, lacks NK cell developmental potential while it maintains the ability to generate helper ILC subsets (ILC1, ILC2, ILC3 and LTi) *(14)*. The CHILP in mice is also referred to as ILCP and can be additionally marked by high PD1 expression *(69)*. NK cell development in mice was initially thought to be independent of the aforementioned intermediate stages as they could branch off at earlier stages of the helper ILC development *(70)*. However, it was recently proposed that NK cells can develop from a common ILC and NK cell progenitor that expresses *Id2 (71)*. These inconsistencies might emerge due varying mouse models or culture conditions.

Similar to murine models, human ILCs develop from multipotent lymphoid-biased progenitors. Although the intermediate differentiation steps from HSC to ILC-restricted precursors are less clearly defined in humans, we have gained substantial knowledge during the last 15 years (Figure 2, Table 1). In 2006, Freud *et al.*, discovered that NK cells originate and develop from $CD34^{+}$ progenitors within secondary lymphoid organs (SLOs), including tonsils and lymph nodes *(72)*. Subsequent studies have since delineated distinct stages of differentiation, so called NK cell developmental intermediates (NKDIs) *(73)*. The first and immature stages of NKDIs were initially described as stage 1 and stage 2, or now called early tonsillar progenitors 1 (ETP1) and 2 (ETP2), defined as $CD34^{+}CD10^{+}CD117^{-}$ (ETP1) and $CD34^{+}CD10^{-}CD117^{+}$ (ETP2) *(68)*. These progenitors were multipotent and capable of generating T cells, NK cells, B cells and dendritic cells (DCs), indicating that more specialized

clones within this pool of cells are present. Subsequently, stage 3 NK cells, minimally defined as $CD34^{-}CD117^{+}CD94^{-}$, lack T cell and DC potential while a small fraction can differentiate to stage 4 'mature NK cells' in response to IL-15 *(73)*. However, the basic phenotype of stage 3 NK cells overlaps with other non-NK ILC subsets like such as ILC3 (as being $CD117^{+}$) *(74)*. This is a topic of discussion regarding the naïve-like ILC subsets in **Paper I** (section 6.1).

A committed NK-cell precursor (NKP), defined as $CD34^{+}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$, was later identified in fetal liver, fetal BM, cord blood and adult tonsils, showing robust NK potential *in vivo* and *in vitro* while lacking capacity for generating T cells, B cells and non-NK ILC *(62)*. Furthermore, a study by Montaldo *et al*. described a population of $CD34^{+}CD45RA^{+}CD117^{+}\alpha4\beta7^{+}CD7^{-}CD127^{-}ROR\gamma t^{+}$ (similar to ETP2) as an ILC3-restricted precursor (ILC3P) in tonsils and intestinal lamina propria *(75)*, that could give rise mainly to mature $ROR\gamma t^{+}$ ILC3, but virtually no $CD94^{+}Eomes^{+}$ NK cells, after co-culture with OP9 stromal cells. However, the capacity to generate ILC1, ILC2, T cells and DCs was not evaluated in this study. Furthermore, studies by Scoville *et al*. identified a human common innate lymphoid progenitor (CILP) in SLOs with the capacity to give rise to NK cells as well as helper ILCs, but not T cells or DCs *(63)*. In addition to the aforementioned ILC3P phenotype, CILP expressed IL-1R1 (receptor for IL-1β), implying an association between inflammatory signals and ILC development within tissues. The identification of tissue-residing precursors suggests that tissue microenvironments might harbor signals necessary for *in situ* ILC development that could ultimately serve as a reservoir for the replenishment of the tissue-resident ILC pool.

Classically, CD34 expression marks early multilineage progenitors as it is also expressed by HSCs. In addition to the $CD34^{+}$ ILCPs that have been described, recent studies have reported novel $CD34^{-}$ ILC developmental intermediates that could replenish the circulating or tissue-resident ILC pool *(38, 76)*. Circulating ILCs are perceived as immature/naïve, with naïve referring to cells that have not been exposed to priming/activation signals *(77)*. Supporting the naivety of circulating ILCs, blood-derived ILCs express fewer genes at the transcriptome level compared to tissue-derived ILCs *(78)*, and do not readily produce cytokines in response to cytokine stimulation *(6, 78)*. Therefore, circulating ILCs could be assumed as the equivalent of naïve T cells, which typically express CD62L and CD45RA. Those surface markers have been described to be expressed by naïve-like ILCs in blood and tonsils *(6, 79)*. In addition to the phenotypic similarities of circulating naïve ILCs and T cells, their developmental trajectories could also be mirrored where circulating ILCs seed organs, differentiate into mature subsets and exert their effector functions. This model of development was proposed in a study from Lim *et al*. whereby a $CD117^{+}$ ILC precursor (termed ILCP) that can seed peripheral tissues and give rise to all ILC subsets, including NK cells *(80)*. Dissecting the heterogeneity of the blood and tonsil $CD117^{+}$ ILC pool further, studies have identified specialized ILC precursors marked by KLRG1, NKp46 and/or CD56 expression, with distinct differentiation potentials. More specifically, blood-derived $CD117^{+}NKp46^{+}CD56^{+/-}$ ILC are able to develop into ILC3, ILC1 and NK-like cells, while $CD117^{+}KLRG1^{+}$ ILCs preferentially differentiate into ILC2 *(38)*. In the tonsil $CD117^{+}CD56^{+}$ ILCs (denoted as rILCP in *(68)*) are able to generate ILC3 and NK-cells, while ILC2s can be generated by $CD117^{+}CD56^{-}$ ILCs *(76)*.

Overall, it is clear that ILC precursor populations deriving from classical CD34+ and non-classical CD34− precursors with uni- or multilineage potential are present in several adult and fetal tissues. In **Paper I** we characterize two tissue-residing precursor-like CD34− populations that potentially represent naïve stages of ILCs (see section 6.1).

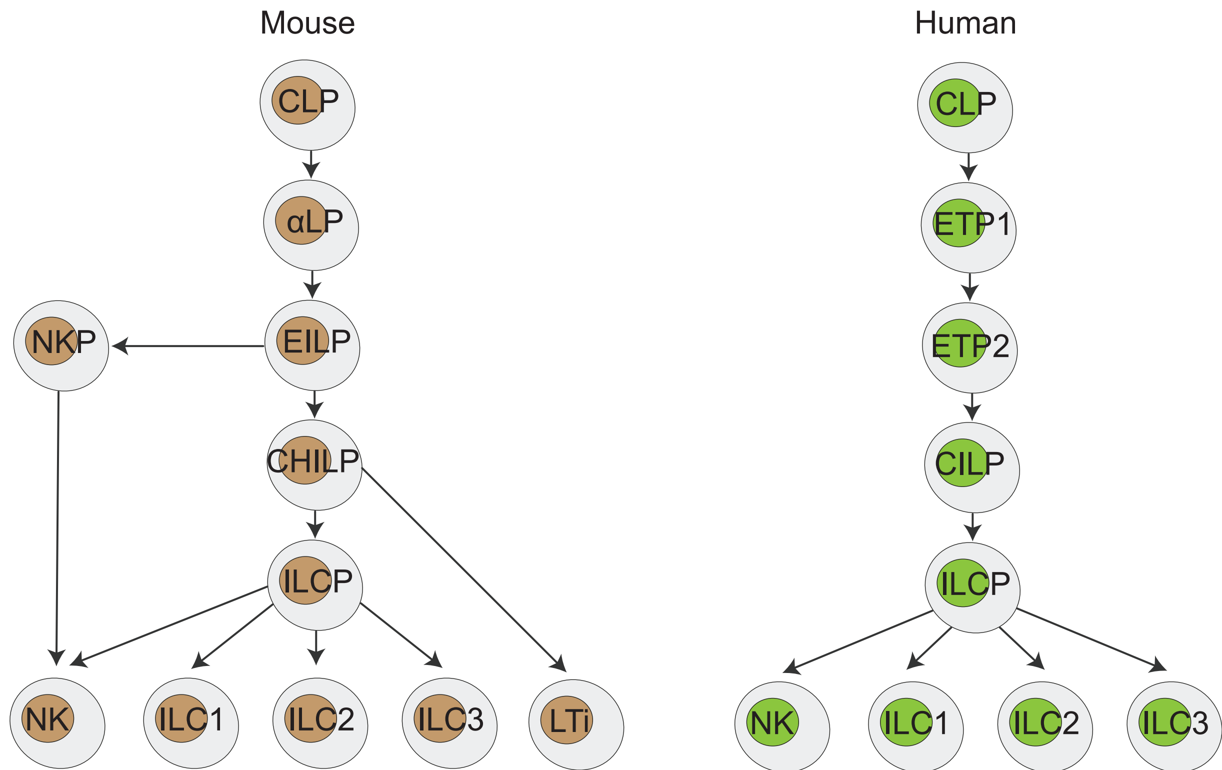

Figure 2. Developmental pathways of murine and human ILC development.

Cell	Identity	Stromal cells	Cytokine stimulation	Time	Subset generation	Ref
ETP1	CD34^{+}CD117^{-}CD94^{-}CD10^{+}	OP9-DL1	IL-3, 7, KL*, FL**, IL-15	28 days	NK, ILC1, ILC2, ILC3,T cells, Myeloid	*(68, 81, 82)*
ETP2	CD34^{+}CD117^{+}IL1R1^{-}CD94^{-}	OP9-DL1	IL-3, 7, KL, FL, IL-15	28 days	NK, ILC1, ILC2, ILC3	*(68, 81, 82)*
Stage 3 NK	CD34^{-}CD117^{+}CD94^{-}	OP9-DL1	IL-3, 7, KL, FL, IL-15	28 days	NK	*(73)*
Stage 4 NK	CD34^{-}CD117$^{+/-}$CD94^{+}	OP9-DL1	IL-3, 7, KL, FL, IL-15	28 days	NK	*(73)*
ILC3P	CD34^{+}CD45RA^{+}CD117^{+} $\alpha4\beta7^{+}$CD7^{-}CD127^{-}RORγt^{+}	γ-irradiated OP9 cells	IL-7, SCF***, FL, IL-15	20 days	NK, ILC3	*(75)*
NKP	CD34^{+}CD38^{+}CD123^{-}CD45RA^{+} CD7^{+}CD10^{-}CD127^{-}	OP9-DL1	IL-3, 7,SCF, FL, IL-15	5 weeks	CD16$^{+/-}$ NK	*(62)*
CILP	CD34^{+}CD117^{+}IL1-R1^{+}CD94^{-}	OP9-DL1	IL-3, 7, KL, FL, IL-15	28 days	NK, ILC1, ILC2, ILC3	*(68, 81, 82)*
ILCP	CD34^{-}CD117^{+}IL1R1^{+}CD94^{-}	OP9, OP9-DL4	IL-2, 7, 23, 1β	14-18 days	NK, ILC1, ILC2, ILC3	*(80)*
n/d	CD34^{-}CD117^{+}KLRG1^{+}	OP9-DL1	IL-2, 7, 23, 1β	7-14 days	ILC2	*(38)*
n/d	CD34^{-}CD117^{+}NKp46^{+}	OP9-DL1	IL-2, 7, 23, 1β	7-14 days	ILC1, ILC3	*(38)*
rILCP	CD34^{-}CD117^{+} IL1-R1^{+}CD56^{-}	OP9-DL1	IL-2, 7, KL, FL	28 days	ILC2, ILC3, NK	*(76)*
rILCP	CD34^{-}CD117^{+} IL1-R1^{+}CD56^{+}	OP9-DL1	IL-2, 7, KL, FL	28 days	ILC3, NK	*(76)*

Table 1. Summary of the human ILC progenitor potential in *in vitro* systems. Adapted and modified from *(68)*. *KL: c-kit ligand, **FL: human FLT3 ligand, ***SCF: c-kit ligand, n/a: not defined.

1.2.1 Role of Notch signaling in ILC development

The Notch signaling is defined as an evolutionarily conserved signaling pathway that is involved in various biological processes including lymphocyte development *(83, 84)*. In mammals, there are four Notch receptors (Notch1-4) and five canonical ligands [Delta-Like (DLL1), DLL3, DLL4, Jag1 and Jag2)] *(84)*.

The Notch signaling pathway has a well-established role in T cell lineage determination. This was reported by a study in 1999 where Notch1 deficient mice were incapable of developing T cells, while generation of other hematopoietic lineages were unaffected *(85)*. Studies later on reported that DLL4 is indispensable for T cell development in the thymus *(86)*, while DLL1 might have a redundant role as it is not expressed by the thymic epithelial cells *(87)*. Interestingly, Jag1 and Jag2 are not implicated in T cell development *(83)*.

In 2002, the laboratory of Dr. Zúñiga-Pflücker developed a mouse bone marrow-derived stromal cell line that ectopically expresses the human DLL1 Notch ligand, so called OP9-DL1. This *in vitro* allowed the study of T cell lineage commitment and differentiation *(88)*. While OP9-DL4 cell line exists, no studies refer to the DL3 ligand as it seems to function as an antagonist *(83)*.

A growing body of evidence has attempted to delineate that role of Notch receptors or the Notch-expressing stroma on the differentiation potential of ILCs. First, a study by Possot *et al*. reported that Notch2 is required for the generation of RORγt$^+$ ILCPs from adult bone marrow, while fetal RORγt$^+$ ILCPs do not necessarily depend on Notch signaling *(89)*. A subsequent study described that the murine CHILP readily gave rise to helper ILC subsets, ILC1, ILC2 and ILC3 when cultured with OP9-DL1 cells *(14)*. In line with the later study, circulating CD34$^-$CD117$^+$ ILCPs that were cultured on OP9-DL4 cell line readily gave rise to all ILC subsets including NK cells in peripheral tissues *(80)*, indicating that indeed the Notch signaling is required for ILC differentiation from precursor cells. Hernandez *et al.* confirmed this idea, whereby OP9-DL1 cells supported the generation of ILCs from cord blood CD34$^+$ HSCs *in vitro (13)*.

Notch2 signaling orchestrates the plasticity between NCR$^-$ ILC3 to NCR$^+$ ILC3 in the mouse lamina propria, and it is required for the maintenance of the NCR$^+$ ILC3 identity *(90)*. Along with the Notch signaling, RORC and IL-23 signals are also essential during ILC differentiation, and specifically ILC3 generation *(91)*.

Several studies suggested that Notch plays a role in NK cells and ILC1s as well. Specifically, a study showed that Notch signaling balances mouse hepatic ILC1 identity by controlling T-bet, while deficiency of RBPJ increases the expression of Eomes in those cells, therefore acquiring a more NK-like phonotype *(92)*. In NK cells, Notch signaling modulates KIR expression and acquisition of effector function *(93)* and it also controls the intermediate stages of human tonsillar NK cell differentiation *(94)*. While NK cells acquire more terminally differentiated stages (stage 4A, 4B) Notch1 receptor increases whereas Notch2 decreases, which is required in earlier NK stages (stage 3) as well as ILC3 and ILC2 differentiation *(94)*. It is evident from other studies that Notch signaling controls ILC2 generation *in vivo* and *in vitro*, and it is attributed to the fact that RORα and TCF-1, two transcription factors that are important for ILC2 development in mice, act as downstream targets of the Notch pathway *(95, 96)*. Lastly, the Notch signal strength works as an equilibrium between T cell (low signal strength) and ILC2 (high signal strength) differentiation *(97)*.

Overall, Notch signaling plays a critical role not only in the differentiation, proliferation and survival of ILCs but can also affect the ILC lineage specification. Hence, in **Paper I**, we used the OP9-DL1 system as a tool for investigating ILC differentiation. It is important to keep in mind that several studies have used either OP9-DL4 or OP9-DL1 to assess differentiation. Whether the two ligands contribute equally to the differentiation potential of ILCs or whether specific ligands favor differently the generation of specific ILC subsets needs to be further investigated.

1.3 PLASTICITY OF HUMAN INNATE LYMPHOID CELLS

Although categorization of ILCs into ILC1s, ILC2s and ILC3s provide a useful global nomenclature for the diversity of ILCs, in complex tissue microenvironments ILCs may display more fluid and diverse states which is reflected by the term ‘plasticity’.

ILC3-to-ILC1 plasticity

ILC3s are the most abundant ILC subset in the human intestine in homeostatic conditions *(98)*, while ILC1s are sparsely present *(12)*. However, studies have shown that in the lamina propria of Crohn’s disease (CD) patients, there is an enrichment of ILC1 and reduction of NKp44^{+} ILC3s *(8, 50, 99)*, implying the possibility for ILC3 to ILC1 plasticity in IBD. Upon intestinal inflammation, there are several cytokine signals released by immune cells that enforce this process. It has been found that IL-12, released by CD14^{+} DCs, and IL-1β by multiple cells, are the signals needed for conversion to occur *(100)*. *In vitro*-cultured intestinal ILC3 with IL-12 plus IL-1β, results in downregulation of CD117 and RORγt and increased production of IFN-γ, reminiscent of an ILC1-like phenotype (so called “ex-ILC3”) *(100)*. Noteworthy, a study has identified intermediate steps between the ILC3 and ILC1 (termed ILC3a and ILC1a respectively in that study) transition in the tonsil and lamina propria *(101)*. These included the CD196^{+}CD300LF^{+} (termed ILC3b) and CD196^{+}CD300LF^{-} (termed ILC1b) subsets, while IFN-γ was gradually increasing from ILC3a to ILC1a via those two subsets *(101)*. Similarly, in the lamina propria an intermediate subset expressing *IL7R*, *CD300LF* and *KLRD1* (CD94) was identified along the ILC3-to-ILC1 trajectory with an ILC1 differentiation propensity *(101)*

Several studies have presented certain transcriptional regulators that might govern the ILC3-to-ILC1 plasticity. Aiolos, a transcription factor (TF) predominantly expressed by ILC1s, was shown to be involved in ILC3 to ILC1 polarization in mice and humans *(102, 103)*. Two mouse studies revealed the importance of the TF c-Maf in maintaining the ILC3 identify through repression of ILC1 signature genes *(104, 105)*, as well as in regulating the ILC1/ILC3 ratio in the mouse intestine *(104)*. Additionally, BCL6 appeared to have repressive effects in ILC1 and ILC3 gene programs and seemed to promote the presence of ILC1s in the mouse intestine *(104)*.

ILC2-to-ILC1 plasticity

ILC2 can exhibit a degree of plasticity in tissues upon inflammatory conditions. In a chronic disease of the lungs such as chronic obstructive pulmonary disease (COPD), ILC2s were able to convert to an ILC1-like phenotype and produce IFN-γ *(106)*. Regarding the cytokines that trigger this process, studies have revealed that similarly to ILC3-to-ILC1 conversion, IL-1β and IL-12 are required *(107, 108)*. For ILC2-to-ILC1 conversion, IL-1β needs to first induce the expression of the IL-12 receptor (IL-12RB) on ILC2 in order to promote IL-12-induced IFN-γ production *(108)*. Similarly, another study of lung and nasal inflammation showed ILC2-to-ILC1 plasticity *in vitro* when ILC2 were stimulated with IL-1β and IL-12, while the presence of IL-4 in the cultures could reverse the conversion *(109)*.

ILC2-to-ILC3 plasticity

Multiple studies report on plasticity of human ILC2 to ILC3-like cells *(110–112)*. CD117^{high} ILC2s in tonsils displayed a more plastic phenotype compared to CD117^{low} ILC2s *(110)*. Epithelial-derived cytokines such as TGF-β, IL-23 and IL-1β are involved in the transdifferentiation of ILC2s into IL-17-producing ILC3s causing their accumulation in nasal polyp inflammation *(111)*. This suggests that IL-17 production might be associated with the pathology of the disease. This is supported by a study in mice which revealed that IL-17 production by ST2$^+$ ILC2 play a pathogenic role in lung inflammation *(113)*. However physiologically, a similar subset of IL-25-responsive KLRG1$^+$ "inflammatory ILC2" (iILC2) were shown to play a critical role in parasite expulsion and *Candida albicans* infection by producing IL-17 *(114)*.

ILC2-to-IL-10 producing ILC2 plasticity

IL-10 producing ILC2s have been detected in the mouse intestine *(115)*. Putative IL-10 elicitors in this study were IL-2, IL-4, IL-27, IL-10 and neuromedin U (NmU), whereas TNF-like ligand 1A (TL1A) acted as a suppressor. In humans, IL-10 producing ILC2s are found in PB and inflamed tissues of patients with chronic rhinosinusitis with nasal polyps (CRSwNP) *(116)*. They could be generated from KLRG1$^+$ ILCs (CRTH2$^+$ or CRTH2$^-$) upon stimulation with IL-33 and retinoic acid (RA). This study suggested that these IL-10-producing ILC2 might contribute to protection against allergic responses.

NK-to-ILC1 plasticity

Plasticity among NK cells and ILC1s has been reported in mice. Several studies have shown that tumor growth factor (TGF)-β drives this conversion, meaning that TGF-β stimulated NK cells could turn into non-cytotoxic ILC1-like cells *(117, 118)*. Mechanistically, SMAD4, a signal transducer of the TGF-β signaling pathway, played a critical role in regulating this process *(117)*. SMAD4- deficient NK cells lost ability to produce cytotoxic molecules and acquired a helper ILC1-like phenotype *(117)*.

ILC3-to-NK plasticity

A study from Raykova *et al.*, demonstrated that human tonsil and humanised mice tissue ILC3 could convert into NK cells when stimulated with IL-12 and IL-15 *(119)*. The differentiated ex-ILC3 displayed the ability to upregulate NK cell-specific markers such as EOMES, CD94, CD56 and CD16 *(119)*. Another study by Hughes *et al. (74)*, which was later confirmed by Montaldo *et al. (75)*, revealed that suppression of the TF AHR results in the conversion of ILC3s to NK cells. This was shown by antagonising or silencing the TF AHR in ILC3 that resulted in the capacity to become CD56^{bright}CD94$^+$IFN-γ-producing NK cells. Adoptive transfer experiments have shown that gut CCR6$^-$NKp46$^-$ ILC3 can convert into IFN-γ producing NK1.1$^+$NKp46$^+$ NK cells *in vivo (120–122)*.

1.4 INNATE LYMPHOID CELL INTERACTIONS WITH T CELLS

ILCs orchestrate immune responses by communicating with other immune cell subsets such as DCs *(123)* as well as myeloid cells *(124)* in a bidirectional manner. ILCs can also interact with adaptive immune cells, including T cells, and evoke enhancive or suppressive immune responses.

The interactions between ILCs and T cells have been primarily studied in mouse models. In mice, ILCs can express MHCII and can through MHCII-TCR interactions *(125, 126)* or cytokine signals *(123, 125, 127, 128)* drive T cell responses. For example, communication between $CD4^+$ Th2 and ILC2 leads to IL-2 production by $CD4^+$ Th2 cells that directly induces ILC2 proliferation and capacity for type 2 cytokine secretion *(125, 126)*. MHCII-expressing ILC2 can also act as antigen-presenting cells (APCs) with the capacity to present antigen to $CD4^+$ T cells *(125)*, and facilitate helminthic expulsion *(126)*.

ILC3s have been also shown to possess antigen-presenting features. Several mouse studies have provided conflicting evidence for MHCII-expressing ILC3 to either limit or promote T cell responses *(129–132)*. For example, it has been shown that intestinal $MHCII^+$ ILC3 are able to provide immune tolerance by suppressing activated commensal bacterial-specific T cells *(129, 130)*. In contrast, another group indicated that interaction of ILC3 and $CD4^+$ T cells leads to proliferation of T cells both *in vivo* and *in vitro (131)*. One possibility for these discrepancies might be due to microbial exposures in different animal facilities. A second possibility is due to the origin of the cells used in those studies. While the first studies investigated intestinal ILCs, the latter demonstrated their findings in splenic and PB ILCs, which are environments with different microbiota landscapes. In fact, it was shown that splenic $MHCII^+$ ILC3 are more potent in inducing $CD4^+$ T cell activation as compared to intestinal $MHCII^+$ ILC3 in mice, further supporting that localization is key to determine their function *(132)*. This was further explained by the low MHCII related transcripts in intestinal ILC3 and the microbiota-induced IL-23 that can reduce MHCII expression in mice *(132)*.

The importance of $MHCII^+$ ILC3 in colonic mucosa was further shown in a study where ILC3 controlled the B cell-derived IgA production, thus maintaining tissue homeostasis *(133)*. However, different microbiota components can induce different effects. For instance, during colitis, microbiota-derived TL1A promotes expression of OX40L in $MHCII^+$ ILC3 that leads to chronic T cell activation *(134)*.

In humans, the crosstalk between ILCs with T cells remains less characterized. Our group, using scRNA-seq identified an *HLA-DR*-expressing ILC3 population in the tonsils *(6)*, indicating the presence of a transcriptionally distinct ILC subpopulation with antigen-presenting properties. For **Paper III**, it was, therefore, natural to explore the possibility of human circulating ILCs ability to present antigens to $CD4^+$ T cells.

2 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a global health issue and its prevalence is increasing in both adult and pediatric patients *(135)*, of whom intestinal samples were analyzed in **Paper I** and **Paper II**, respectively. Pediatric IBD (pIBD) is defined as being diagnosed before the age of 18 years, a group that makes up 25% of all IBD patients *(135)*. IBD is characterized by chronic inflammation of the GI tract and its cause is multifactorial, where genetics, diet, lifestyle and immune response are some of the factors involved *(136)*. In this thesis, we have focused on understanding immune mechanisms that might be responsible for driving the disease pathogenesis.

IBD consists of two types, CD and Ulcerative colitis (UC), that differ in the location and the depth of inflammation in the intestinal wall. CD is characterized by patchy transmural inflammation with granulomas and can affect any part of the GI tract, from mouth to anus *(136)*. On the other hand, UC is characterized by diffuse mucosal inflammation and it affects only the large intestine, from caecum to rectum *(137)*.

Adult and pediatric patients with IBD display differences in the presentation and disease course *(138)*. PIBD is characterized by a more extensive disease at onset compared to adults, including more frequent pancolitis in pediatric UC *(138)*. Additionally, pediatric patients are often diagnosed at an earlier phase of the disease than adults, which typically live with symptoms for a longer period or are more prone to seek health care at a later phase. Pediatric patients are also less likely to be affected by environmental factors such as smoking and air pollution or medications of any kind. For the aforementioned reasons, it makes it important to investigate IBD mechanisms in both age groups.

2.1 TREATMENT OPTIONS IN IBD

The treatment of IBD has been relying on immunosuppression, with administration of steroids and immunomodulators (azathioprine, 5-ASA) in the initial phase of the disease *(139)*. Later on, and as soon as the disease progresses, more specific immunosuppressive drugs (biologics) are introduced. These drugs target pro-inflammatory cytokines such as the anti-tumor necrosis factor (TNF)-α with anti-TNF antibodies (e.g infliximab, adalimumab), IL-12/23 with anti-IL-12/IL-23 antibody drug (ustekinumab) or adhesion molecules with anti-α4β7 integrin antibody drug (vedolizumab) that blocks the trafficking of lymphocytes in the intestine *(139)*.

Other attempts to block proinflammatory cytokines such as IL-17 resulted in paradoxical effects. Specifically, blockade with IL-17 inhibitors (e.g secukinumab, brodalumab etc.) in clinical trials exacerbated the disease. This was further explained by a growing body of evidence that IL-17 is important for maintaining intestinal barrier integrity *(140)*.

2.2 INNATE LYMPHOID CELLS IN IBD

ILCs typically reside in mucosal barrier sites, including the intestine. The intestine is largely divided into small (including the ileum) and large intestine (including the caecum, ascendance, transverse, descendance, rectum). Although ILCs are present in all these intestinal anatomical regions, slight differences in their composition occurs. For example, the ileum has been shown to contain significantly increased frequencies of ILC1s, ieILC1s and NKp44^{-} ILCs as compared to the colon regions, while the NKp44^{+} ILC3 frequencies are greater in the colon but still the dominant population throughout the gut *(141)*.

Studies using intestinal samples from adult IBD patients have demonstrated that the composition of ILCs in the inflamed IBD is altered with increased frequencies of ILC1s and reduced frequencies of NKp44^{+} ILC3s *(8)*. In mice, several models of colitis have demonstrated a tissue-protective role of ILC3 *(100, 142, 143)*. Mice lacking ILC3 developed severe colitis in DSS colitis model compared with mice that had ILC3 *(144)*. IL-22, a signature cytokine produced by NKp44^{+} ILC3s, has a well-established role in intestinal tissue protection. Some of the reasons are 1) induction of mucous proteins and antimicrobial peptides (AMPs) *(145)*, 2) protection and activation of intestinal stem cells *(146)*, 3) regeneration of intestinal epithelium after tissue damage *(146)*, 4) promoting epithelial cell fucosylation (glycosylation mechanism of host-microbiota symbiosis) *(147)*. Classically, IL-23 promotes IL-22 production by ILC3s. However, in experimental mouse models of colitis, IL-23 treated ILC3s contributed to pathology via production of IL-17A, IFN-γ and IL-22 *(148, 149)*.

The increase of ILC1s can be derived due to conversion of ILC1-to-ILC3 in both mice *(14)* and humans *(100)*. IL-12 and IL-1β are likely the drivers since human ILC3s can differentiate to IFN-γ-producing ILC1s when cultured with these cytokines *(100, 150)(151)*. Although studies report increase in the conventional CD127^{+} ILC1s located in the lamina propria *(8)*, studies have also shown increase in ieILC1s able to release granzymes and perforin in both mice *(18)* and humans *(8)*. A similar CD127^{+} CD94^{+} ILC1 population that shares characteristics of both CD127^{+} ILC1s and CD94^{+} NK cells has been found to increase in the intestinal lamina propria of CD patients *(152)*. Interestingly, upon IL-15 stimulation this population is able to produce perforin and granulysin, the latter an antimicrobial protein, while their cytotoxic effector molecules are suppressed in the presence of TGF-β *(152)*. Reports on the frequencies of NK cells in IBD are conflicting. While several studies present higher frequencies of NK cells in IBD colonic lamina propria *(153, 154)*, other studies found decreased frequencies of NK cells in inflamed IBD biopsies *(141)*, as well as in blood of CD patients *(155)*.

The role of ILC2s in IBD remains unclear, largely due to the absence of conventional CD127^{+} ILC2s in the human adult gut *(12, 32)*, which could also be related to the gut microbiota. This is supported by the fact that ILC2s are present in fetal gut *(156)* where microbiota is absent, as well as in antibiotic-treated mice *(157)*. However in mice, IL-33 treated ILC2 provide intestinal protection through production of amphiregulin (AREG) *(158)*. Exposure to IL-33 and RA, mouse intestinal can also release IL-10, which is interesting in light of its anti-inflammatory

effects *(115)*. Since ILC2s in the mouse intestine represent a major subset, it makes it difficult to directly translate findings of mouse models concerning intestinal ILC2s in humans. Frequencies of ILC2s were elevated in the intestinal mucosa of IBD patients *(50)*, however it is possible that they accumulate in the lamina propria due to the general lymphocyte influx upon inflammation *(159)*. Further studies are needed to fully uncover the role of these cells in IBD.

While most studies concerning IBD analyze intestinal samples, blood samples from these patients are also worth investigating for the identification of potential biomarkers. Indeed, signalling lymphocytic activation marker 1 (SLAMF1), which is an inhibitory molecule, is increased in expression in circulating ILC2 in UC and CD patients, making this molecule an interesting therapeutic target *(160)*.

Taken together, ILCs play a significant role in IBD considering their capacity to respond to environmental signals and release effector molecules. Understating the inflammation-specific role of ILCs in the intestine could pave the way for the identification of more effective and treatment strategies.

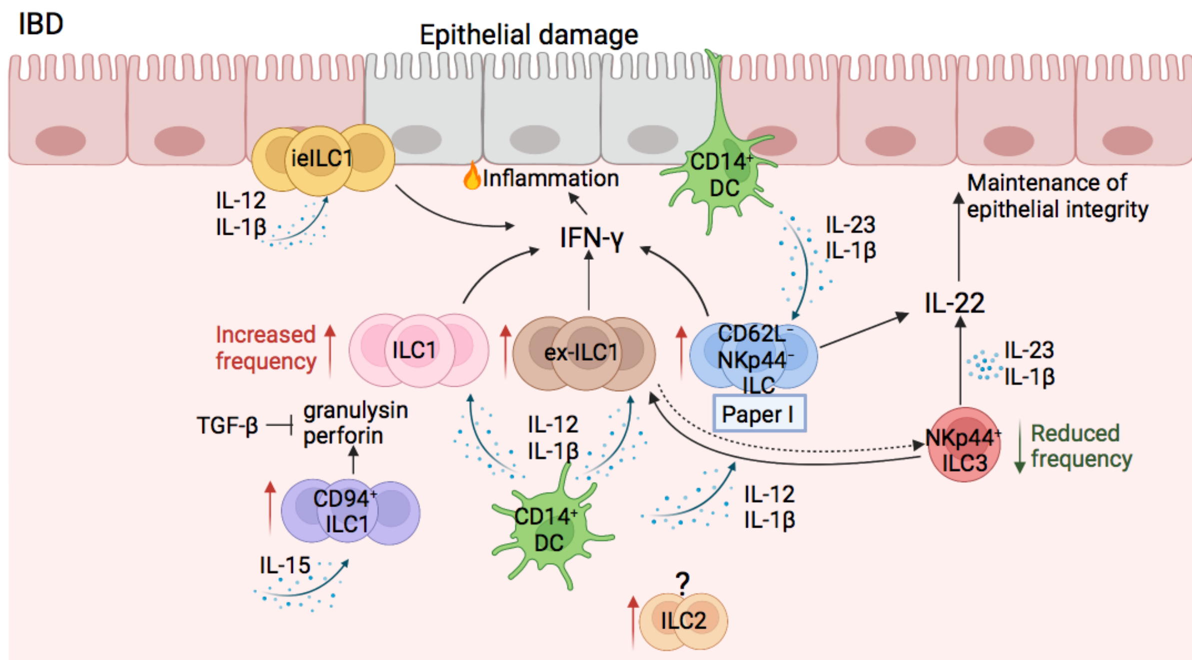

Figure 3. Graphical illustration of the human ILC plasticity in IBD. Figure designed with BioRender.

3 INNATE LYMPHOID CELLS IN COLORECTAL CANCER

Although ILCs have been implicated in several types of cancer, patients with IBD have a high risk of developing colorectal cancer (CRC) *(161, 162)*. A growing body of evidence has suggested that ILCs are implicated in pro-tumoral and anti-tumoral responses depending on the ILC subset and the type of cancer *(163)*.

NK and ILC1

First, NK cells have a well-established role in cancer using different co-stimulatory and inhibitory molecules as well as cytotoxic molecules, perforin and granzymes to kill target cells *(164)*. However, their role in CRC is poorly understood. A study reported that NK cells are sparsely infiltrated in colorectal carcinoma tissue *(165, 166)*, although they represent together with ieILC1s 95% of the tumor-infiltrating ILCs *(12)*. Furthermore, Coca *et al*., analyzed 157 CRC patient samples and found that patients with high infiltration of $CD57^+$ NK cells have a longer survival and a favorable tumor outcome than those with lower infiltration *(167)*.

Scarce is the data for the role of ILC1s in CRC as well. A study investigating the peripheral compartment of metastatic CRC found that $CD56^+$ ILC1 cells are expanded whereas the rest of the ILC subsets are reduced *(168)*. The accumulation of $CD56^+$ ILC1s was associated with lower antitumoral Th1 response *(168)*, suggesting that these cells play a pathogenic role in metastatic CRC. Conversely, an antitumor *SLAMF1*-expressing CRC-specific ILC1 subset was detected in scRNA-seq data from CRC tumors *(169)*, suggesting that ILC1 could also contribute to a better CRC outcome.

ILC2

Since ILC2s are largely absent from the human intestine, the role of ILC2 in CRC is yet unclear. However, recent findings demonstrate a potential anti-tumoral role of ILC2 in CRC *(170)*. ScRNA-seq analysis identified an ILC2-resembling cluster in CRC tumors compared with the healthy gut *(169)*, thus implying their capacity of ILC2 to infiltrate tumors. In fact, ILC2 with anti-tumor properties have been found to infiltrate human and mouse melanoma tumors *(171)* as well as hepatocellular carcinoma *(172)*. Moreover, tumor-derived IL-33 triggers IL-13 production by ILC2s which is sustained by peroxisome proliferator activated-receptor gamma (PPAR-γ) *(173)*. Conditional deletion of PPAR-γ in ILCs is show to reduce cancer development and proliferation in a murine CRC model *(173)*. Another mechanism of IL-33 being pro-tumoral is by promoting colon cancer cell growth and recruitment of macrophages to enhance colon cancer carcinogenesis *(174)*. Further research is need to fully characterize the role of ILC2 in CRC.

ILC3

As previously mentioned, ILC3s are the dominating ILC subset in the intestine, thus, understanding their role in CRC is undoubtedly crucial. Several studies using mouse models have thus far collectively shown that ILC3s infiltrate tumors and affect the cancer progression

mainly via cytokine production *(175–181)*. One of the early studies that described the role of ILC3s in intestinal cancers was from Kirchberger *et al*. in 2013, showed that bacterial-induced colon cancer was driven by IL-22-secreting ILCs in mice *(179)*. This was explained by the capacity of IL-22 to bind the IL-22 receptor (IL-22R) on dysplastic colonic epithelial cells and promote their proliferation (and therefore cancer progression) in a Stat3-dependent manner *(179)*. The involvement of Stat3 activation in triggering excessive IL-22 production was further confirmed by another study in human colon cancer *(182)*, which additionally showed elevated IL-22 serum levels detected in CRC patients *(183)*. Given that ILC3s are instrumental in keeping gut homeostasis, it is possible that the effector functions of ILC3s are impaired by the tumor microenvironment. Supporting this hypothesis, studies have shown that ILC3s are diminished and dysregulated in CRC tumors, with a subsequent increase of ILC1s *(169, 184)*, implying increased plasticity between the two subsets. Importantly, previous data has suggested a role for ILC:T cell interactions in CRC *(184)*. Although HLA-DR^{+} ILC3s were reduced in pediatric CD *(130)*, HLA-DR expression on ILC3s from CRC tumors was increased compared to non-malignant adjacent tissues *(184)*, indicating a potentially different role of these cells in the two diseases. In a mouse model of CRC, depletion of MHCII^{+} ILC3 resulted in invasive CRC and resistance to anti-PD1 immunotherapy *(184)*. This study *(184)* demonstrated the importance of the crosstalk between ILC3 and adaptive immunity in CRC, which is a concept that we further investigated in **Paper III**.

In summary, ILCs play an important role in CRC and could be utilized for improved therapeutic strategies.

4 RESEARCH AIMS

The overarching aim of this thesis is to better understand the biology of ILCs in homeostasis as well as in the intestinal diseases IBD and CRC.

The specific aims of this thesis are:

- **Paper I**: Determine the transcriptional and chromatin-accessibility landscape as well as the differentiation capacity of naïve/quiescent-like ILCs in tonsillar tissue and intestinal tissue of patients with IBD.

- **Paper II**: Dissect the transcriptional landscape of ILCs and T cells in the colonic mucosa of pediatric patients with IBD.

- **Paper III**: Unravel the antigen-presenting properties of human blood and intestinal HLADR^{+} ILCs.

5 METHODOLOGICAL CONSIDERATIONS

In order to experimentally address our research questions and hypotheses, several relevant methodologies were used that are discussed in this section. The studies were conducted *ex vivo* (analysis of cells that are directly extracted from the living body) and *in vitro* (analysis of cells in a laboratory dish) using various techniques including flow cytometry/fluorescence-activated cell sorting (FACS) (**Papers I**, **II, III**), bulk (**Paper I**) and single-cell RNA sequencing (**Papers I**, **II**) and assay for accessible chromatin followed by sequencing (ATAC-seq) (**Paper I**). All these methods were used to characterize the phenotypic, functional, transcriptional or chromatin states of ILCs and gain a deeper insight into the biology of ILCs as well as other lymphocyte populations. Experiments and methodologies were performed on precious human material and, therefore, ethical considerations should be discussed.

5.1 ETHICAL CONSIDERATIONS

All studies included in this thesis were conducted using human samples and performed in accordance with the guidelines described in the Declaration of Helsinki. Prior to using the samples, all donors provided informed consent signed by themselves or from their legal guardians (in case of children). To ensure anonymity of donors' personal information, all samples were coded with only age or gender information available tonsil donors. For gut samples, we additionally had information on their medical treatment or diagnosis. Blood samples were received completely anonymised. In case of the gut samples, information on the age, gender and medication were important and facilitated the research and data analysis performed.

More specifically, in **Paper I**, we used tonsils, blood, gut resection specimens and gut biopsies. The tonsils were derived from patients with obstructive sleep apnoea (OSA) where removal of tonsils was part of the clinical treatment procedure. Hence, this material does not impose any extra burden to the patients since tonsils would be otherwise discarded. Buffy coats, that were used in **Papers I** and **III**, came from blood donations collected at the Blood Central, Karolinska Hospital, Huddinge. Due to our scientific question, in **Paper III**, we were specifically interested in knowing the cytomegalovirus (CMV) status of the buffy coats. Other than that, we had no information regarding the name, gender or age of the donors. Therefore, such samples raise low ethical concerns.

In **Papers I**, **II** and **III**, I used intestinal biopsies or resection specimens. In **Paper I**, I received non-inflamed and inflamed gut specimens or biopsies from adult patients with IBD that needed operation for removing the inflamed part of the colon/ileum. As part of the standard surgery procedure, the diseased piece as well as the tissue specimen from the non-inflamed area were resected and further used in my research. All tissue resections were done for medical treatment or diagnostic purposes and no additional tissue was resected for research purposes. Similarly, in **Paper III**, we used non-affected and tumor specimens (tumor and tumor border) from

patients undergoing colorectal cancer surgery. Overall, those gut samples are part of the clinical procedure and, thus, do not impose additional suffering for the patients.

In **Paper II**, gut biopsies from pediatric donors with IBD were used. These samples were collected during diagnostic endoscopy after receiving permission from their legal guardians. The non-IBD donors that served as controls in **Paper II**, were patients that showed gastrointestinal symptoms and colonoscopy was performed to rule out the diagnosis of IBD. Biopsy collection for research purposes, in addition to the rutine diagnostic biopsies, can marginally increase the lenght of the endoscopy and anesthesia. Besides the extra time needed, patients should experience no additional pain. However, there is a minor risk for extra bleeding. The overall goal using this unique human material is to find novel therapies for the treatment of IBD.

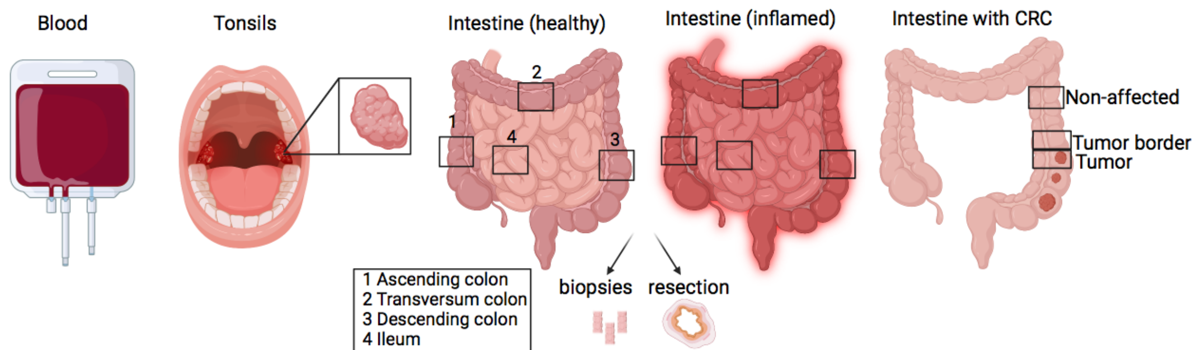

Figure 4. Summary of the samples used in this thesis. Figure designed with BioRender.

5.2 FLOW CYTOMETRY AND CELL SORTING

Flow cytometry is a technique that allows for immunophenotyping of large number of cells at the single-cell level. In principle, each single cell is analysed for light scatter, which provides information on the size of the cells, and one or multiple fluorescent parameters *(185)*. The cells are stained with fluorescently-labelled antibodies against surface or intracellular molecules and, subsequently, fluorescence is measured by the cytometer's lasers (excitation optics) *(185)*. Because ILCs are fairly rare and are distinguished by excluding lots of other cell lineage markers, flow cytometry is a powerful and important technique that allows ILC research through phenotypic labelling of markers specifically expressed by them. In my studies, flow cytometry was primarily used for 1) measurement of frequencies of immune cells (**Papers I**, **II**, **III**) 2) evaluation of expression levels of surface and intracellular proteins (including secreted cytokines) *ex vivo* and upon stimulation (**Papers I**, **II**, **III**), 3) functional assessment of ILC and T cell responses to antigen stimulation (**Paper III**).

In addition to ILCs, in my studies I used flow cytometry to immunophenotype T cells and NK cells derived from blood, tonsils or intestinal tissues. These three cell populations show overlapping phenotypes and functions that can be captured by flow cytometry. In my studies I have used up to 18-parameter flow cytometry. Although modern flow cytometers can measure 50 parameters simultaneously, one drawback of this method still remains, and it is the limitation in the number of markers one can investigate in the cells. Therefore, flow cytometry, although it provides meaningful data, is limited in capturing heterogeneity within cell populations.

FACS is based on flow cytometry where in addition to immunophenotyping, live cells can be sorted according to the expression of labelled proteins, in either tubes or plates. This gives the ability to isolate cells of a specific phenotype and research *ex vivo* or *in vitro* their properties. FACS has been a key method in all three papers of this thesis, since it allowed me to enrich for rare ILC subsets in tonsils and gut and investigate them at bulk and single-cell level.

5.3 GENE EXPRESSION AND EPIGENETIC ANALYSIS

Nowadays, sequencing technologies allow for capturing genome-wide expression in biological samples. In my studies, we used a variety of different methods including bulk RNA sequencing (RNA-seq), single-cell RNA sequencing (scRNA-seq) and assay for transposase accessible chromatin (ATAC-seq) to transcriptionally profile bulk or single cells as well as to assess the chromatin accessibility throughout the genome.

5.3.1 Bulk RNA-sequencing

Bulk RNA-seq is a powerful method that uses sequencing to measure the level of messenger RNA (mRNA) expression in cell populations present in biological samples. With this technique cDNA libraries from sort-purified cell populations are sequenced and aligned to the human genome, thus providing information on the genes that are being transcribed (Figure 5). More specifically, in **Paper I**, we performed bulk RNA-seq of different tonsillar ILC subsets to compare their transcriptomes and unravel distinct transcriptional signatures. Although it is a very useful technique, bulk RNA-seq is looking at the transcriptome across whole cell populations, thus limiting information on individual cells.

5.3.2 Single-cell RNA sequencing

Compared to bulk RNA-seq, single-cell RNA sequencing analyses the transcriptomes of individual cells, thus providing information on cellular heterogeneity within biological samples. We used two scRNA-seq methods that differ in chemistry and gene capture. In **Paper I**, we used a SMART-seq2 scRNA-seq dataset of ILCs from non-affected non-IBD and inflamed adult colon, previously generated by Mazzurana *et al.* 2021 *(4)*. SMART-seq2 technology captures the full-length mRNA, providing the advantage of capturing rare

transcripts or investigating gene isoforms or gene splicing. In **Paper II**, we generated a new scRNA-seq dataset with a droplet-based method (10X Genomics), which partitions individual cells into bead droplets that are uniquely barcoded to index each cell's transcriptome (Figure 5). Our goal using this method was to characterize rare cell populations and cell states that are otherwise impossible to identify with bulk sequencing. Thus, it paves the way for determining novel cell subsets and transcriptional networks with the help of computational analysis. However, it is important to keep in mind that transcription of a gene does not necessarily result in protein translation. It is therefore important to consider validation of scRNA-seq experiments on a protein level. Nowadays, techniques such as e.g CITE-seq enable RNA sequencing along with qualitative and quantitative information on surface proteins in single cells.

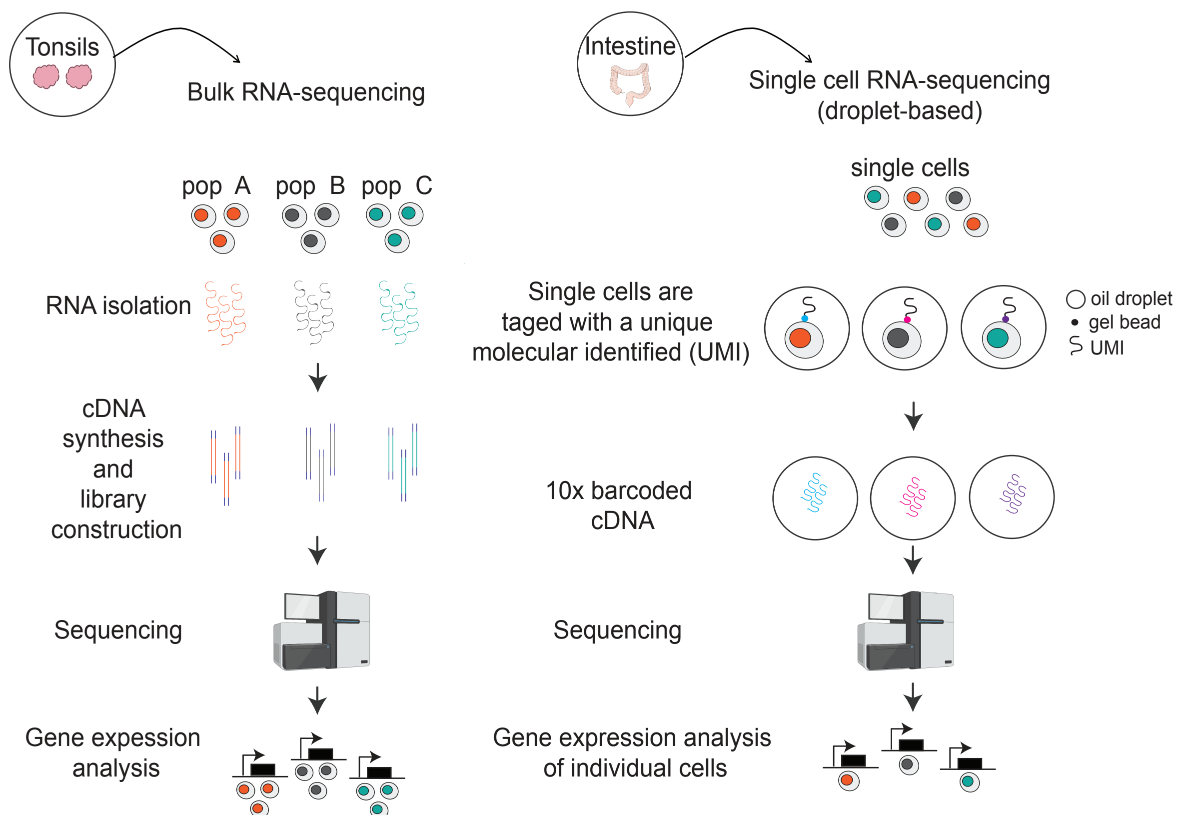

Figure 5. Graphical illustration of the bulk RNA-seq and scRNA-seq (10X Genomics) experimental process.

5.3.3 ATAC-sequencing

Transcriptional profiling indicates what genes are being transcribed by the cells. However, it does not inform on the expression potential of poised genes at resting or activating state. Assay for Transposase Accessible Chromatin using sequencing (ATAC-seq) that was first described by Buenrostro *et al.*, 2013 *(186)*, is an epigenetic analysis that profiles the sites of open chromatin where active transcription (or suppression) can take place. An enzyme called

transposase 5 (Tn5) is inserted to cleave off the regions where chromatin is open followed by amplification and sequencing of the libraries (Figure 6). By performing this technique in **Paper I**, we aimed to understand the epigenetic state of the naïve-like ILC subsets in comparison to the differentiated subsets. This method also enables identification of transcription-factor motif imprints, which are genomic sequences that specifically bind to transcription factors.

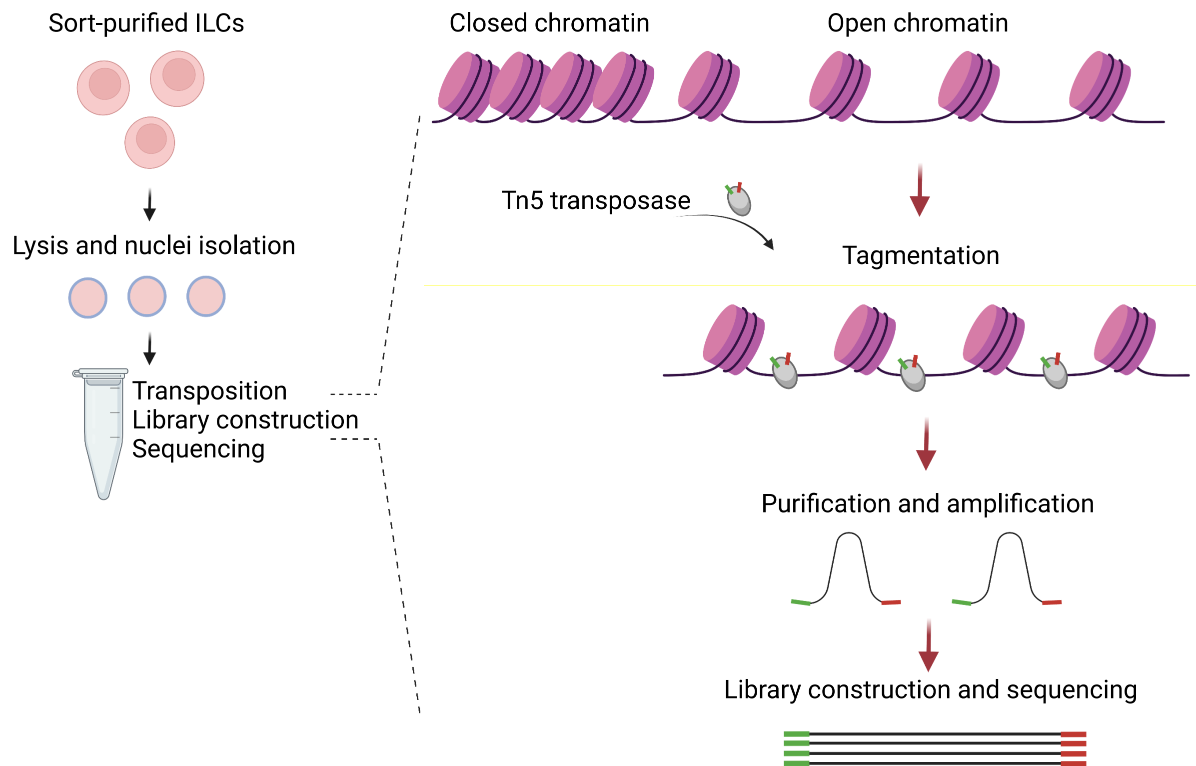

Figure 6. Workflow for performing ATAC-seq of ILCs. Figure designed with BioRender.

5.4 OP9-DL1 *in vitro* system

The OP9-DL1 is a bone marrow-derived mouse stromal cell line that expresses the delta-like 1 (DL1) notch ligand. This cell line has been traditionally used to study lineage commitment and differentiation of T or B cells, since notch signaling is important in providing the appropriate survival and proliferation signals. Lately, OP9-DL1 or OP9-DL4 has been used to explore ILC differentiation as well, as performed in **Paper I**. More specifically, in **Paper I** we sort-purified bulk or single cell ILC subsets and seeded them on top of OP9-DL1 stroma, in order to evaluate their differentiation capacity (Figure 7).

While this culture system enables the study of lymphocyte differentiation, one should keep in mind that it is an *in vitro* system and does not necessarily mimic the *in vivo* conditions.

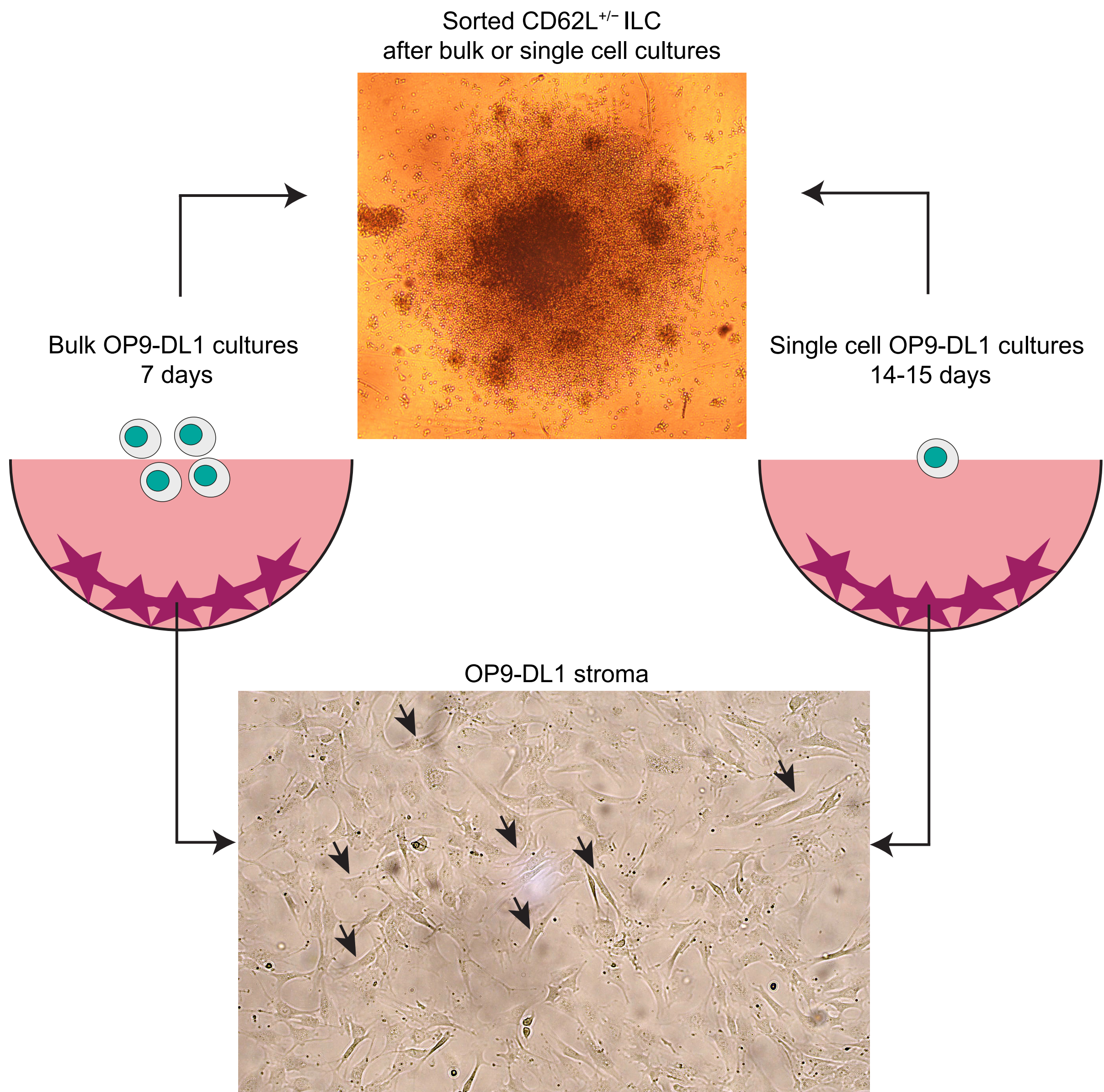

Figure 7. Schematic diagram and morphology of the ILC:OP9 stomal bulk and single cell co-culture system. Microscopy pictures taken with a camera microscope by Efthymia Kokkinou.

6 RESULTS AND DISCUSSION

6.1 PAPER I – DIFFERENTIATION POTENTIALS OF NAÏVE-LIKE ILCs IN HUMAN MUCOSAL TISSUES

In **Paper I** we aimed to understand the differentiation capacity of naïve-like ILCs in human tissues.

The first evidence of the presence of naïve ILCs in tissues was presented in 2016 by Björklund *et al.* where the heterogeneity of tonsillar ILCs was described in depth using scRNA-seq *(6)*. Three distinct ILC3 subpopulations were identified. One cluster showed enrichment of the *NCR2* transcript (encoding NKp44), which was an already known subset by the time, the second cluster was dominated by *HLA-DR* transcripts, while cells in the third cluster were smaller, had fewer transcripts and expressed *SELL* (encoding CD62L) *(6).* Typically, naïve T cells are marked by CD62L and CD45RA expression *(187)*. Given that this subset of ILCs expressed both of these two markers and were transcriptionally and functionally less active *(6)*, it raised the possibility that it represents a naïve ILC population.

The work presented in this paper additionally builds upon previous studies by Lim *et al. (80)*, Nagasawa *et al. (38)*, and Chen *et al. (76),* that collectively deepen our understanding of the model of human ILC development and differentiation from CD34^{-} precursor-like cells.

We dissected the tonsillar (CD34^{-}CD94^{-}CD117^{+})NKp44^{-} ILC fraction with regards to protein expression of CD62L and CD45RA. We revealed the presence of three subsets of CD62L^{+}CD45RA^{+} ILCs (denoted as CD62L^{+} ILC), CD62L^{-}CD45RA^{+} ILCs (denoted as CD62L^{-} ILC) and CD62L^{-}CD45RA^{-} ILCs (Paper I, Fig. 1A). This landscape varied significantly in blood. Bulk RNA-seq demonstrated clearly that the tonsillar CD45RA^{+} ILCs subsets have distinct transcriptional profiles between them and as well as when compared to ILC1, ILC2, NKp44^{+} ILC3 and CD62L^{-}CD45RA^{-} ILCs (Paper I, Fig. 1C). The latter subset displayed great transcriptional and functional similarity with NKp44^{+} ILC3s, indicative of its more differentiated state.

Of note, the phenotype of the CD45RA^{+} ILC subsets (CD34^{-}CD94^{-}CD117^{+}NKp44^{-}) is reminiscent of the stage 3 NK cells. As discussed in section 1.3, stage 3 NK cells are a heterogenous group of cells that potentially includes ILC3s *(74)*. It is, therefore, possible that the CD45RA^{+} ILCs are part of this pool of 'stage 3' cells that are further distinguishable by CD62L and CD45RA expression. Some of the similarities between stage 3 NK cells and CD45RA^{+} ILCs are the fact that both can generate NK cells *in vitro* when cultured with IL-15 and IL-12/IL-1β, respectively. However, unlike stage 3 NK cells, CD45RA^{+} cells potentially generate immature NK cells (Paper I, Fig. S3B, NKG2A and CD94 expression). Stage 3 NK cells are characterized by unique LFA-1 expression and produce IL-22 *ex vivo*, while CD45RA^{+} ILCs can produce little to no IL-22 without stimulation. LFA-1 expression by CD45RA^{+} ILCs was not tested. Overall, the lineage relationships between these subsets remains to be further understood.

Next, we observed transcriptional differences in terms of the metabolic pathways the $CD45RA^+$ ILC subsets engage. For example, $CD62L^+$ and $CD62L^−$ ILCs were low in transcripts related to glycolysis and fatty acid metabolism, which are known to be utilized by activated T cells to fulfil their needs *(188)*. Conversely, oxidative phosphorylation (OXPHOS), typically utilized by naïve T cells *(189)*, was enriched in $CD62L^+$ ILCs, further supporting its naïve state (Paper I, Fig. 1E-G). In line with this, we showed that conversion from a naïve to a differentiated and functional ILC state depends on metabolic reprogramming and utilization of glycolysis (Paper I, Fig. 6). Similarly, a recent study reported that differentiation into an ILC1-like phenotype by lung precursors in the mouse is controlled by the glycolytic program *(190)*.

Analysis of the chromatin state was able to provide further clues regarding the status of the cells. ATAC-seq indicated $CD62L^+$ and $CD62L^−$ ILCs have distinct global epigenetic profile from the differentiated subsets. Additionally they lack TF motifs associated with differentiated ILC subsets (RORC, TBX21, GATA3) (Paper I, Fig. 3B-D). Therefore, their epigenetic profiles further supported the naïve state of those subsets.

Having established that these cells represent naïve/quiescent states of ILCs, we further wanted to explore whether this was reflected in their functionality. Indeed, short-term cultures (12hr) with OP9-DL1 and cytokines showed their inability to efficiently produce key ILC effector proteins (IL-22, IFN-γ and IL-13) (Paper I, Fig. 2D-F). Of note, the $CD62L^−$ ILC showed signs of IL-22 production, potentially indicating that it is an intermediate, less naïve, subset. Next, we asked whether a longer time period would allow them to differentiate into other ILC subsets. Indeed, we found that 7 days in culture with OP9-DL1 and cytokine stimulus polarized them to IL-22- and IFN-γ-producing cells, acquiring an ILC3 and ILC1-like phenotype, respectively. Interestingly, ILC2 differentiation was not evident in these cultures. One possible explanation is that this is due to very low KLRG1 expression (Paper I, Fig. S3C) which is known to mark ILC precursors prone to ILC2 differentiation *(38)*. Another possibility is the variable expression of CD56 (Paper I, Fig. S3C) especially considering that $CD56^−$ ILCPs favor ILC2 development *(76)*. To define heterogeneity of the $CD62L^+$ and $CD62L^−$ ILC fate potential, we also performed single cell cloning assays where we found functional multi-potency towards ILC1 and ILC3 subsets (Paper I, Fig. 5).

To investigate the role of these subsets in the context of IBD, we first wanted to know whether such subset exists in the gut. ScRNA-seq revealed the presence of a transcriptionally distinct cluster of quiescent/naïve-like ILCs in the colon (cluster 0) defined by *SELL*, *TCF7* and lack of genes related to differentiated ILC lineages (*NCR2*, *RORC*, *TBX21*) (Paper I, Fig. 7A), suggesting that we might have identified a subset similar to what we had already described in the tonsil. In fact, we found that gut quiescent/naïve-like ILC cluster displayed high transcriptional similarity with the tonsil $CD45RA^+$ ILC subsets and shared genes that were collectively associated with cellular quiescence (Paper I, Fig. 7E-G).

To validate by protein the existence of such a subset, we analyzed the frequencies of the naïve-like and differentiated ILC subsets in intestinal IBD resections. First, we confirmed that the frequency of $NKp44^+$ ILC3s in inflamed IBD is reduced, as previously reported *(8, 50)*, while

there was a parallel increase of the CD62L⁻ ILC subset (Paper I, Fig. 8D-E). Different scenarios could explain such accumulation; 1) recruitment from blood as a result of the general lymphocyte infiltration in IBD *(159)* or as proposed by Lim *et al.* *(80)* 2) local proliferation, however we detected low Ki67 expression *ex vivo* (Paper I, Fig. S5F), or 3) they derive from differentiated ILCs that have lost their function. Such possibility was evident in a model of psoriasis whereby ILC3 converted to ILC2 via a quiescent ILC stage *(191)*. To test if the CD62L⁻ ILC subset in the inflamed intestine was capable of ILC differentiation we performed single cell cloning assays. This experiment interestingly showed that *in vitro* this quiescent subset acquired effector functions since a considerable amount of clones had high propensity for IL-22 production (Paper II, Fig. 8I-J). The landscape of effector function acquisition was quite different from the phenotypically similar subset in the tonsil where most clones produced IFN-γ (Paper I, Fig. 5D and 8J). This implies that potentially the tonsil and intestinal microenvironment imprints the CD62L⁻ ILC subset in a different way and also that the potential loss-of-function of mature tissue-resident ILC3s could be restored *in vitro*. The human intestine is highly dominated by ILC3s, therefore, it could be hypothesized that progenitors in the human intestinal environment might have a stronger propensity to generate the subset they are mostly comprised of, as a way to keep the homeostasis. The preferential IL-22-producing ILC3 differentiation in the gut is important in the context of IBD, as it can be a promising target for the promotion of tissue repair in the intestine.

Overall, in **Paper I**, we describe naïve stages of ILCs defined by CD62L and CD45RA expression. In the tonsil the double expressing cells, called CD62L⁺ ILCs, represent the most naïve form of ILCs while the cells that lack CD62L expression potentially represent a more quiescent, intermediate stage of ILCs. In the intestine, a similar quiescent/naïve like CD62L⁻ ILCs subset is present in increased frequencies. Functionally these cells can differentiate to IL-22-producing ILC3s and ILC1s. These findings expand on our knowledge of the 'plastic' players in IBD and potentially lay the foundation for discovering new therapies.

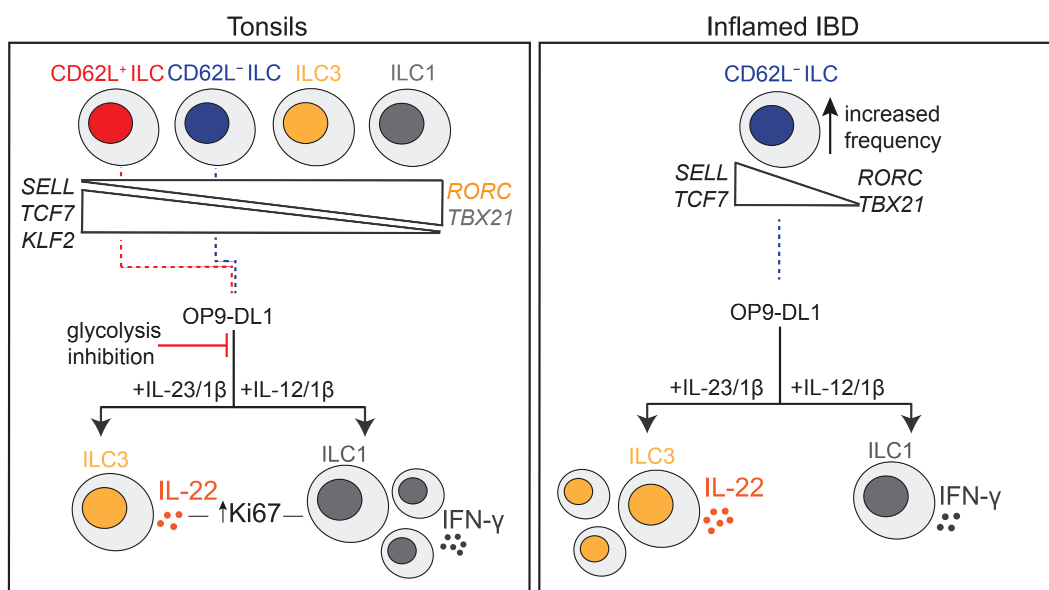

Figure 8. Graphical summary of Paper I.

6.2 PAPER II – TRANSCRIPTIONAL LANDSCAPES OF ILCS AND T CELLS IN PEDIATRIC IBD

IBD is a multifactorial disease that affects people in adolescence or early adulthood (<18 years, referred to as pediatric patients) as well as adults (>18 years). Although ILCs have been investigated in adult patients with IBD *(8, 50)*, whether these changes are similar or different in pediatric patients is not yet known.

In **Paper II,** we performed immunophenotypic and transcriptional characterization of ILCs in pediatric UC and CD. In addition, the transcriptome of ILCs was in-parallel compared to T cells and NK cells. It should be noted that the ages of the patients in our study ranged from 10 to 17 years. Thus, the primary cause of IBD in these patients is unlikely due to genetic mutations in single genes (monogenetic IBD), such as in the IL-10 pathway (*IL10*, *IL10RA, IL10RB*), which is a cause of severe colitis during the first weeks of life *(192)*. However, this age group, compared to adults, allows for studies of disease mechanisms that are less influenced by environmental factors (e.g smoking), co-morbidities and medications. Of note, all pediatric patients in our study were newly diagnosed and treatment-naïve. For flow cytometry analysis we also included non-IBD age-matched patients as healthy controls, because non-inflamed intestine from IBD patients could be partly affected as shown in adult patients *(50)*. Although biopsy collection from healthy pediatric individuals is difficult, the patients included in our study had general gastrointestinal symptoms, thus, they underwent colonoscopy to rule out the IBD diagnosis.

In IBD, the severity of inflammation is measured by endoscopic and histological findings. Endoscopic inflammation was assessed with the SWIBREG (Swedish Inflammatory Bowel Disease Register) scoring system, that takes into account various macroscopic (visual) signs to measure the degree of inflammation in a scale of 0-3 (Paper II, Table S2-S3). Additionally, we evaluated histological inflammation with a modified version of the D'Haens score, which in fact measures the acute and chronic cellular changes within the intestine (Paper II, Table S4).

Immunophenotypic analysis of ILCs with flow cytometry indicated that their frequency during pIBD is altered (Paper II, Fig. 1B). Specifically, the frequency of ILC1s and NKp44^{-} ILCs was increased, while NKp44^{+} ILC3 was decreased in the inflamed IBD as compared to non-IBD controls. A similar pattern has been found in adult IBD patients *(50)*, indicating that inflammation causes a similar effect in both age groups. A reduction of the IL-22 producing NKp44^{+} ILC3s potentially leads to the 'leaky gut' phenomenon in IBD as IL-22 contributes to the protection of the epithelial barrier tight junctions *(193)*, while the increase in ILC1s might be the result of the ILC3-to-ILC1 plasticity, as previously described *(100)*. We also looked at the frequency of the naïve/quiescent-like ILCs we describe in **Paper I**, and found, in line with adult IBD, that pediatric IBD is also characterized by an increased frequency of CD45RA^{+}(CD62L^{-}) ILCs (Paper II, Fig. 4J). Whether these changes are the cause or consequence of the disease pathogenesis is still not clear. However, understanding more about

these compositional changes might provide evidence on the mediators that are involved and pave the way for the development of novel therapeutic targets.

Interestingly, the frequency of ILC1, ILC2 NKp44^{-} ILCs and CD45RA^{+} ILCs was positively correlated to both endoscopic and histological scores, whereas the frequency of NKp44^{+} ILC3 was negatively correlated, meaning that the extent of inflammation impacts the ILC composition (Paper II, Fig. 4K). Collectively, ILC frequency in pIBD is dysregulated and strongly influenced by the extent of inflammation.

To assess transcriptional changes of innate and adaptive lymphocytes in high resolution, we performed single-cell RNA sequencing with the 10X genomics platform. More specifically, we analyzed the transcriptomes of 44717 single cells, including CD127^{+} ILCs, CD56^{+}CD127^{-} NK cells and CD3^{+} T cells isolated from the endoscopically least and most inflamed areas of the colon of treatment-naïve pediatric patients with CD colitis (Paper II, Fig. 2A).

First, a UMAP plot visualized all the cells we had initially sort-purified, including one cluster of ILCs, two clusters of NK cells and 9 clusters of T cells, including CD4^{+} T and CD8^{+} T cell subsets (Paper II, Fig. 2B-C).

To gain insight into the transcriptional relationships between ILCs and T cell subsets, we performed weighted gene co-expression network analysis (WGCNA). This bioinformatic tool constructs gene sets (modules) that are stratified based on their gene expression level, and thus, genes present within modules are highly correlated *(194, 195)*. We generated 50 modules, 11 of which were further explored in the study. Modules that were unique to ILCs or T cells or shared between ILCs and T cells were detected. We focused on certain modules uniquely expressed by ILCs, in an effort to unravel novel gene networks that dictate their function. Module one contained genes encoding for effector molecules such as *IL22*, *CSF2* (encoding GM-CSF) together with *IL4I1*, which works as an metabolic immune checkpoint that enhances AHR activation *(196)* (Paper II, Fig. 3C). Interestingly, although the human intestine lacks ILC2s, we identified a module (11) that contained transcripts known to regulate ILC2/Th2, such as *IL4R* and *CYSLTR1* (encoding the cysteinyl leukotriene receptor) as well as *SOX4* which has been shown to suppress Th2 differentiation *(197)*. This indicates the potential presence of ILC2s in the human intestine and further investigation is needed to fully unravel markers for their identification in the human intestinal microenvironment.

Although it is well known that ILCs and T cells highly overlap in phenotype and function, we know little about the transcriptional programs they share. We found that ILCs and T cells have coordinated gene expression of transcripts related to tissue residency (*FOS*, *NR4A1*, *NR4A2*), antigen presentation (*HLA-DRA*, *HLA-DPA1*), cellular quiescence (*TSC22D3*, *DUSP1*, *ZFP36L2*, *KLF6*) and naivety (*KLF2*, *TCF7*) (Paper II, Fig. 3E-I). This suggests cooperation and reciprocal functional regulation of ILCs and T cells that could be either complementary or redundant during an immune response. More specifically, we found that several of these genes were confined to specific subsets of ILCs when further ILC clustering was performed (Paper II, Fig. 4). Additionally, in **Paper I**, we identified clusters of ILCs that potentially represent

quiescent-like and naïve ILCs in pIBD; one was defined by *SELL* expression (cluster 1) and one contained transcripts such as *TSC22D3*, *DUSP1*, *CD69* (cluster 4), that were previously reported to be expressed by quiescent ILCs in a mouse model of psoriasis *(191)* (Paper II, Fig. 4A-B, E-G). Collectively, we identified previously unknown transcriptional networks that are either unique to ILCs or shared with T cells. This sheds light onto the functional crossroads of these two highly related subsets.

To better understand the cellular changes induced by intestinal inflammation in pIBD, we used a bioinformatic tool of neighbor-based smoothing to more precisely detect the most inflamed and least inflamed cells in our dataset. This method takes into account the Euclidean distance between the cells (neighbors), assuming that close-in-proximity cells display similar functions *(195, 198)*. To perform this analysis, we found it more appropriate to consider the histological inflammation score of the samples as it reflects the acute and chronic changes of the epithelium. In fact, our samples consisted of various histological scorings (Paper II, Table S5), creating a spectrum of inflammation that spanned from low (>4), intermediate (5-8) and high degrees of inflammation (<9) (Paper II, Fig. 5E). To evaluate the differences in the least inflamed and most inflamed cells, we further focused on the low (>4) and high (<9) histological scores and we found an altered cellular landscape in inflammation (Paper II, Fig. 5F-G). ILCs, $CD4^+$ and $CD8^+$ Tn/cm and innate-like $CD8^+$ Trm cells with a 'canonical' transcriptional signature were present in the least-inflamed group while NK cells, cytotoxic $CD4^+$ T cells, activated $CD4^+$ T/Tregs and $CD8^+$ Teff cells were present in the most inflamed group (Paper II, Fig. 5H-I). At least two scenarios could explain these cellular changes; 1) influx of cells in the intestine during inflammation (which is common in IBD) *(159)* or 2) that the least inflamed cells undergo plasticity and give rise to inflammatory cells, resulting in this skewed landscape. Further research is required to delineate the cause of compositional changes, possibly with lineage-tracing experiments that track the migration, proliferation and differentiation of specific cell subsets *in vivo (199)*. Additionally, protein validation of the transcripts detected in DE analysis in the most and least inflamed subsets is essential to potentially find new functions.

Collectively, our work provides the first characterization of ILCs in pediatric IBD as well as unravels transcriptional programs that potentially coordinate ILC and T cell functions in the gut during IBD.

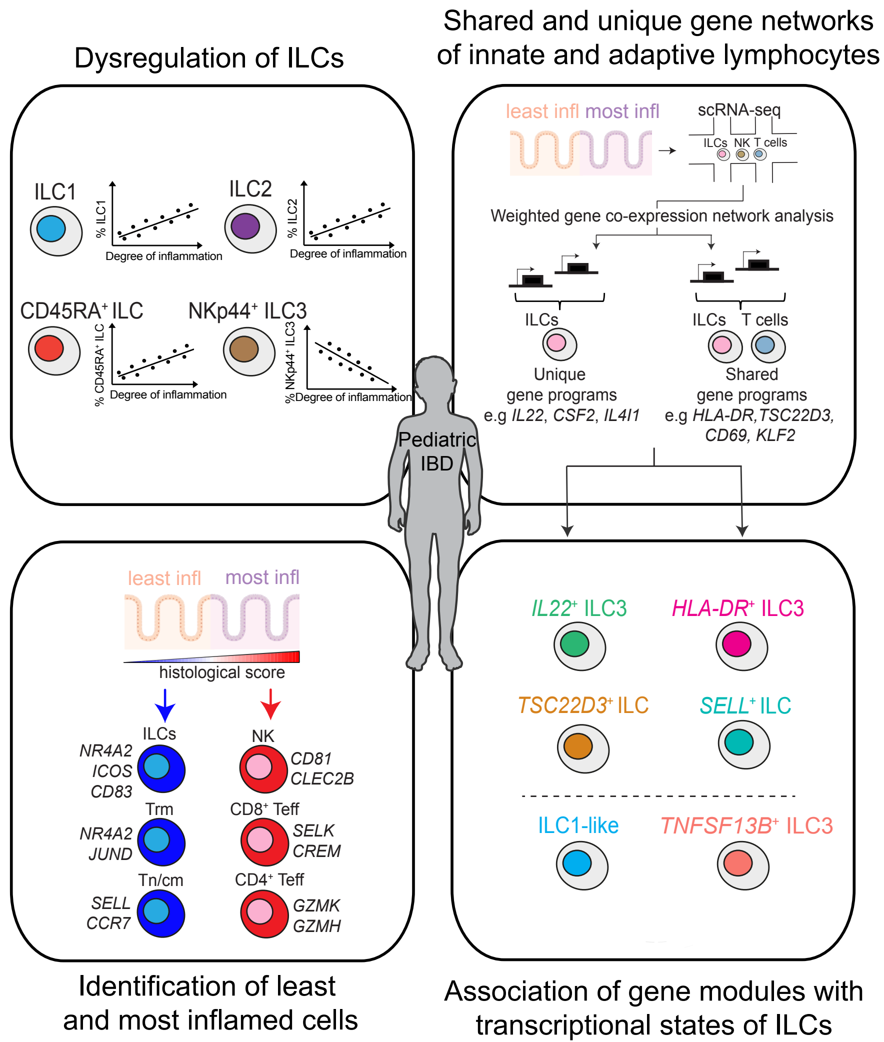

Figure 9. Graphical summary of Paper II.

6.3 PAPER III – ANTIGEN PRESENTING CAPACITIES OF CIRCULATING AND INTESTINAL ILCS

As discussed in section 1.4, ILCs have been shown to communicate with T cells. Although several studies have investigated the MHCII-mediated crosstalk between ILCs and T cells in mice, such interaction in humans is not fully characterized. **Paper III** builds on a previous tonsil scRNA-seq dataset that demonstrated the presence of a distinct *HLA-DR*-expressing ILC subset *(6)*, suggesting the capacity for antigen presentation. Therefore, the work presented in this paper is devoted to understanding the functional characteristics of this subset within healthy and CRC tissue.

First, we analyzed the HLA-DR expression dynamics in ILCs in tumor and tumor border colon specimens from CRC patients as well as non-affected intestine. We observed elevated HLA-DR expression in the tumor tissues compared to control (Paper III, Figure 1A-B). HLA-DR is a key molecule classically used by professional APCs, like DCs, MΦs or B cells in order to present peptide antigens to CD4^{+} T cells and eventually elicit an immune response *(200)*. HLA-DR can be also expressed by T cells, where it is recognized as a marker of activation *(201)*. In order for antigen presentation to occur, close proximity of ILCs and T cells *in situ* is required. Using confocal microscopy we observed that the presence of HLA-DR^{+} ILCs in both non-affected colon tissue were in proximity to T cells (Paper III, Fig. 1C-D), which further supported the notion that ILCs might be capable of presenting antigens to T cells *in vivo.* Due to limitations in performing *in vivo* experiments in humans, to further explore the antigen-presenting features of ILCs, we used PB ILCs as a model to test our hypothesis. More specifically, we deployed an *in vitro* system described elsewhere *(202)*, where we addressed the possibility of ILCs inducing memory CD4^{+} T cell responses to human cytomegalovirus (CMV) (Figure 10). In that model selection of memory CD4^{+} T cells was chosen, since compared to naïve T cells, they might not be in need of co-stimulation to recall a response *(203)*. Designing and developing this protocol was the part that I contributed to the most in this paper.

Although we had established that PB ILCs are able to uptake and process full length protein and express HLA-DR *ex vivo* (Paper III, Fig. 2), these cells were unable to induce CD4^{+} T cell recall responses without prior stimulation (Paper III, Fig. 3). This suggested to us that certain cytokine signals might be responsible for regulating this process. In fact, the tonsillar *HLA-DR*-expressing ILC subset was enriched in transcripts encoding the IL-1β receptor (*IL1R1*) *(6)*. Stimulation of ILCs with IL-1β resulted first in the upregulation of HLA-DR and co-stimulatory molecules (CD80, CD86 and CD70) (Paper III, Fig. 4) via NFκB signaling (Paper III, Fig. 5) and further in the induction of recall responses in CMV-pp65-specific CD4^{+} T cells through the production of IFN-γ, TNF cytokines (Paper III, Fig. 6B-C). This was in line with a previous study, where IL-1β upregulated MHCII and co-stimulatory molecules in splenic ILC3, which enabled them to promote proliferation of naïve T cells *(131)*. In parallel, we analyzed the effects of IL-18 which is a cytokine closely related to IL-1β, however no synergistic effects became evident. The process of antigen presentation by ILCs was halted in the presence of TGF-β (Paper III, Fig. 6F). TGF-β is a crucial regulator of gut immunity which

usually originates in response to the microbiota and has potent immunosuppressive effects *(204)*. This raises the possibility that the human gut microenvironment could potentially suppress the antigen presentation by ILCs.

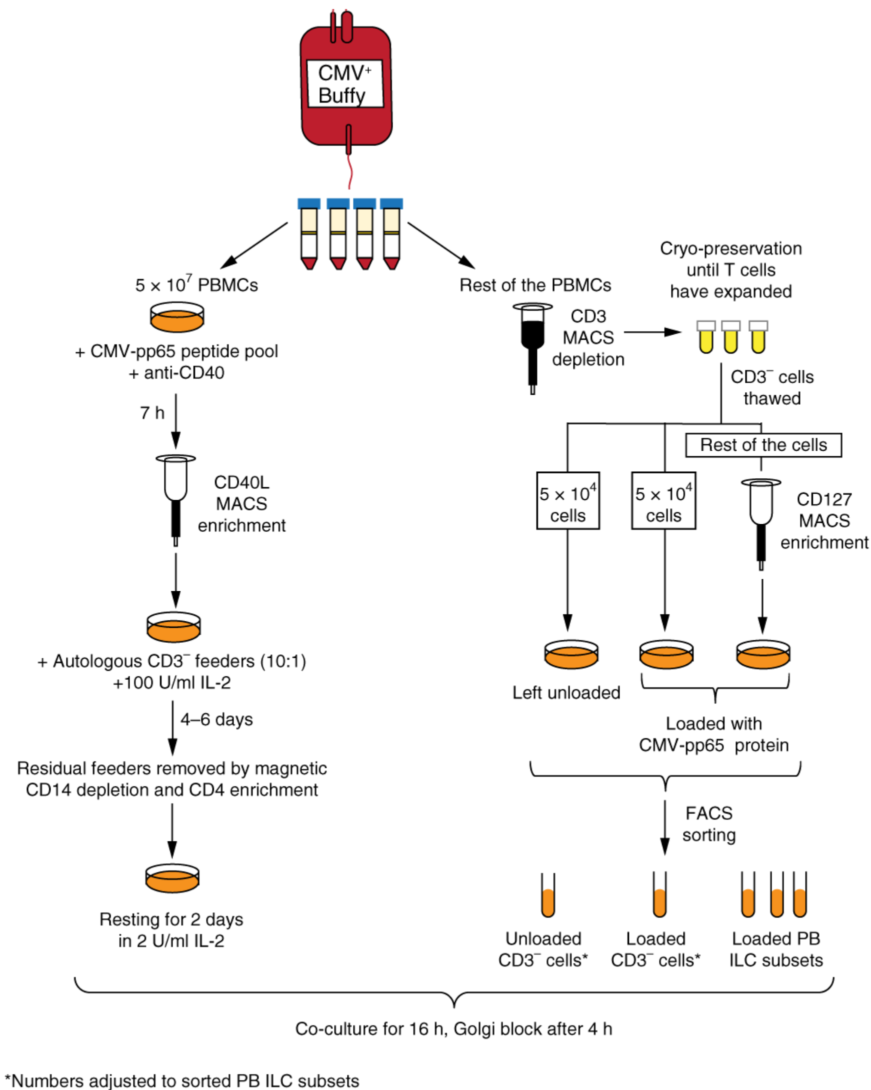

Figure 10. Experimental workflow for addressing the possibility of ILCs to present antigen to CMV-pp65-specific CD4^{+} T cells (Paper III, Fig. 3A).

When we analyzed ILCs from intestinal CRC specimens, we found that stimulation with IL-1β/IL-18 upregulated HLA-DR and co-stimulatory molecules (even to a higher extent than their PB counterparts) (Paper III, Fig. 8B). Whether these tissue-resident HLA-DR^{+} ILCs have a stimulating or activating ability towards CD4^{+} T cells in the tumors needs further investigation.

Overall, this *in vitro* model of PB ILC:T cell cultures allowed revealed that ILCs can adopt APC functions and identify possible networks of CRC-related (IL-1β, TGF-β) cytokines that could regulate antigen presentation of ILCs in the intestinal microenvironment. The potential redundant or compensatory effects of the antigen-presenting ILCs with other APCs in the intestinal mucosa remains to be further understood.

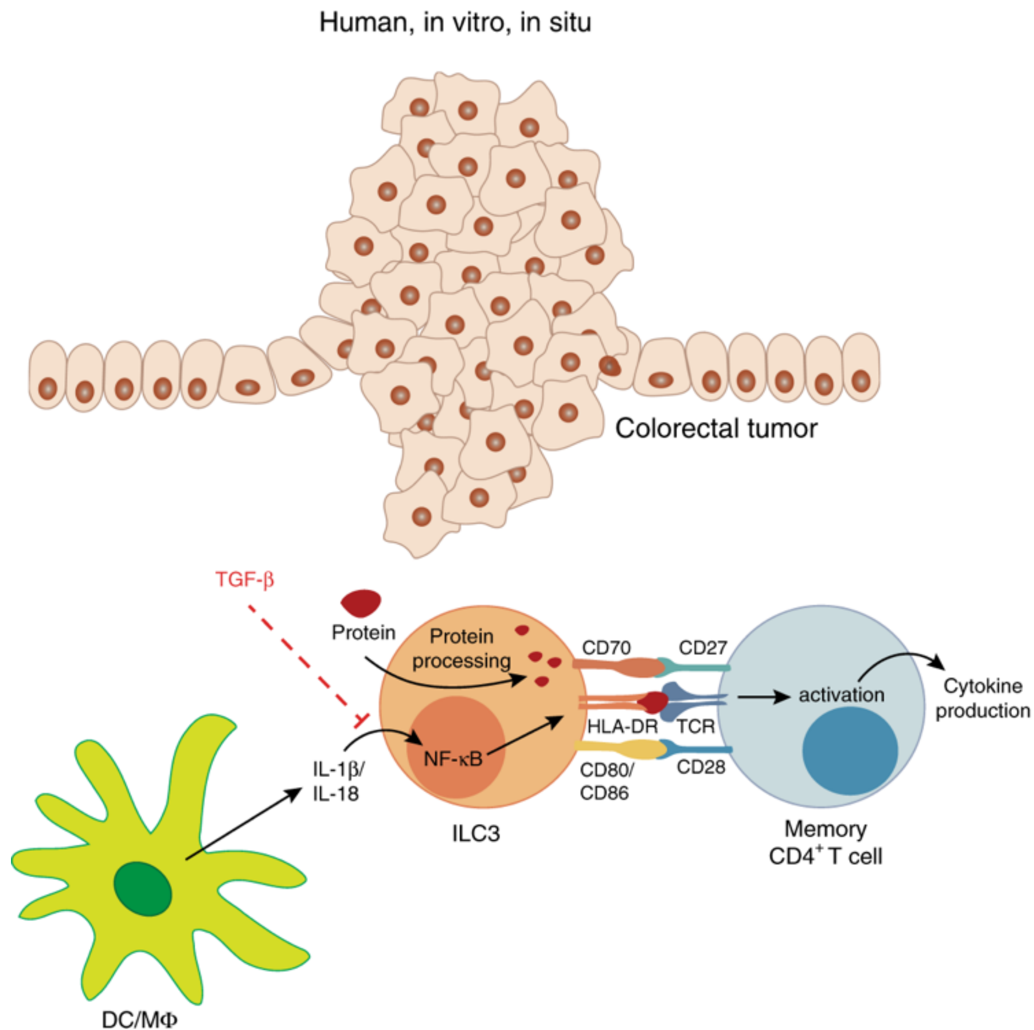

Figure 11. Graphical summary of Paper III.

7 CONCLUDING REMARKS

Throughout the past decades substantial efforts have been made to expand our understanding on the development and functions of ILCs. Although a lot of the current knowledge stems from animal experimentation that can provide *in vivo* biological relevance, translational ILC research is crucial when knowing that the mouse and the human system differs This thesis, performed solely on human samples, attempts to bring forward some of the open questions in the field as well as open new avenues of ILC research. The key findings of my thesis work are:

1. In **Paper I**, we describe the presence of two subsets of ILCs with naive features in healthy and inflamed primary tissues, which could be marked by CD45RA and differing CD62L expression. Although previous studies have reported the presence of naïve ILCs in tissues, this paper provides identification markers and functional properties of these cells in tonsils and the intestine. Most importantly, we describe a novel plastic player of $CD62L^{-}$ ILCs that accumulates in the gut of IBD patients and has the capacity to release IL-22. These findings expand our knowledge on ILC regulation and diversification in IBD and identify new possible targets to restore tissue-immune homeostasis.

2. In **Paper II**, we characterized for the first time the ILC compartment in pediatric patients with IBD. We found that ILCs are altered in composition in inflamed IBD as compared to non-inflamed and non-IBD tissue, including increased frequencies of ILC1s, $NKp44^{-}$ ILCs, $CD62L^{-}$ ILCs and reduced frequencies of $NKp44^{+}$ ILC3. Moreover, all subsets were correlated to the severity of the disease. This indicates that dysregulation of ILCs could be involved in the pathogenesis of IBD. Furthermore, we focused on the transcriptionally shared and distinct programs that underlie ILC and T cell function. We showed that ILCs and T cells display similarities in tissue residency, naive and antigen presention-related transcripts. This provides a useful resource for further unraveling the common and distinct functions of these two systems that could give clues for their regulation in health and disease.

3. In **Paper III**, we addressed the antigen presenting properties of human ILCs. We found that $HLADR^{+}$ ILCs are able to present antigen to memory $CD4^{+}$ T cells and this process is regulated by environmental cues such as IL-1β and TGF-β. Additionally, we showed that $HLADR^{+}$ ILCs are located in close proximity to T cells in the colon, raising the possibility of ILCs regulating T cell responses *in vivo*. This work unravels the APC features of ILCs in the intestinal microenvironmnet which opens new avenues of exploring whether such interaction has a suppressing or activating effect in the human intestine and whether such crosstalk could potentially be harnessed for therapeutic strategies against colorectal cancer.

7.1 FUTURE PERSPECTIVES

1 – **Paper I** opened new avenues in terms of the metabolic pathways ILCs engage in order to execute their properties. Understanding further tissue metabolism and how it influences the cell's properties, we might be able to unleash unprecedented functions of ILCs within tissues or manipulate their functions to control disease.

– The identification of the CD62L^{-} ILCs in tissues also needs further investigation.
1) It would be interesting to explore whether the presence and the properties of such cells can predict responses to medical drugs, such as in cohort of patients on biological drugs e.g ustekinumab, infliximab that are currently used to treat IBD.
2) Using the *in vitro* OP9-DL1 system, it would be interesting to investigate whether the ILCs generated from the naïve precursors acquire a tissue-resident phenotype. This would provide insights into the regulation of tissue-residency and quiescence by ILCs.

2 – In **Paper II**, it would be interesting to include non-IBD controls in the scRNA-seq dataset for a better 'healthy' reference. As scRNA-seq methods keep improving, a potentially more advanced method that would include the performance of V(D)J recombination analysis would be interesting to do. Furthermore, comparing pediatric vs adult IBD single cell transcriptomes could be a useful follow up in order to unravel gene signatures specific to the age groups, if any. 'Pediatric gene signatures' could be potentially more closely related to the mechanisms involved in the initiation of the disease.

3 – In **Paper III**, performance of spatial transcriptomics of colonic HLA-DR^{+} ILCs would be an interesting follow up. Knowing that HLA-DR^{+} ILCs exist in intestinal tissues and are spatially close to T cells, this technique would generate important information on gene signatures linked to their location and potentially function.

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And so? What would follow after completing my Master’s degree? Well before I complete it, I started emailing research labs that I was interested in continuing my training at. One of them was Jenny Mjösberg’s lab ☺ To be honest at that time I had no idea what an ILC was :D Nevertheless, I got a reply from her within 5min and, to cut the long story short, I started as a research assistant in her lab in June 2016 and as a PhD student in 2017.

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