Characterization of human innate lymphoid cells (ILCs) in umbilical cord blood

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„The power to be strong and the wisdom to be wise,
All these things will come to you in time,
On this journey that you are making,
there will be answers that you seek,
and it’s you who climbs the mountain,
it’s you who reached the peak …”
Son of man, Phil Collins

Summary

Innate lymphoid cells (ILCs) have been described to be important players within the innate immune system able to protect mucosal barriers and maintaining tissue homeostasis. However, most insights on ILC biology and function were mostly gained by analysing tissue ILCs, while knowledge on circulating ILCs in particular neonatal ILCs is still scarce. In this thesis neonatal ILCs were first characterised by development of an 8-colour flow cytometry panel with a lineage cocktail designed to exclude unwanted cell populations within umbilical cord blood (CB). Once this panel was established, neonatal ILCs were tested for functionality and it became obvious that two ILC subsets - ILC1 and ILC3 - are unresponsive to the described specific cytokine stimulation indicating different functional properties compared to tissue ILCs. As ILC1 were the least well described ILC subset and were thought to be closely related to NK cells, research first focussed on the analysis of ILC1 in comparison to NK cells. Our data show that CB ILC1, here referred to as ILC1-like cells, express several T cell-associated genes such as *CD5, CD6, CD28* as well as variable regions of the TCR alpha and beta chains (*TRAV/ TRBV*). Since the majority of ILC1-like cells did not exert typical ILC1-related effector functions, we hypothesized that they might be an immature cell type. Based on the observation that ILC1-like cells express certain chemokine receptors and that they show a significant reduction in frequency and total cell count with increasing gestational age, we next hypothesized that they potentially migrate into secondary lymphoid tissues for further maturation. Indeed, when subjecting single cell or bulk ILC1-like cells to conditions favouring NK cell development (seeded on the murine stromal OP9-DL1 and in the presence of cytokines), ILC1-like cells differentiated into $NKG2A^{-}KIR^{+}$ NK cells, representing an advanced stage of NK cell development. Of note, in conditions favouring T cell development no up-regulation of CD3 was observed but increased cell death was noticeable, indicating lack of T cell potential. A complex KIR repertoire with down-regulation of NKG2A was only observed within *in vitro* cultured ILC1-like cells, but not $CD56^{bright}$ NK cells cultured in parallel. ILC1-like derived NK cells were phenotypically as well as functionality similar to *ex vivo* $CD56^{dim}$ NK cells due to their intracellular expression of perforin and granzyme B, ability to mobilize cytotoxic granules, and their potential to kill HLA-I-deficient cell lines as well as kill target cells via antibody dependent cell cytotoxicity (ADCC). Hence, our data demonstrated that CB ILC1-like cells constitute a novel NK cell precursor (NKP).

The next step was to analyse the transcriptomic signature of CB ILCs. A clear distinction between CB ILCs and NK or T cells were detectable, although CB ILCs -in particular ILC1-like cells- clustered more with T cells, while CB ILC3-like cells clustered more with NK cells. Interestingly, CB ILCs shared a distinct set of differential up-regulated genes compared to $CD56^{bright}$ NK cells: the chemokine receptor *CCR4, CD28, SLAMF1*, and the transcription factor (TF) *ID3*. Notably, we observed an unusual *ID3/ID2* ratio > 1, which was only seen within CB ILCs and not seen within CB NK cells, tonsillar ILC3 or peripheral blood (PB) ILCs, taken from a published data set. Interestingly, this unique ratio was also seen within CB $CD4^{+}$ T cells, but not in CB $CD8^{+}$ T cells or PB $CD4^{+}$/ $CD8^{+}$ T cells possibly indicating a shared developmental pathway of CB ILCs and $CD4^{+}$ cells. We further observed novel differentially expressed genes within each ILC subset, such as the TF *ZBTB46* for ILC3-like cells.

We next focused on ILC3-like cells. Although ILC3-like cells exhibited no functional capacity (except for LIF production) when stimulated with specific cytokines, we searched for

alternative stimulatory pathways. We identified exclusive expression of TLR2 and TLR1 chains on ILC3-like cells, but not on other ILCs, and indeed observed a significant higher proliferation rate in ILC3-like cells stimulated with the TLR2:1 ligand Pam_3CSK_4 including high secretion of various cytokines indicating a vital role of ILC3-like cells in host innate immune defence pre-birth.
We also focussed on the *in vitro* generation of ILC3, since tissue-resident ILC3 were previously described to support gastrointestinal tissue homeostasis via secretion of IL-22. Their clinical use is however currently hampered by the lack of suitable cell sources: on the one hand access to human tissue ILC3 is ethically difficult as well as logistically challenging, on the other hand circulating ILC3-like cells do not correspond functionally to their respective tissue counterparts. We thus explored the possibility to generate human ILC3 with clinically important features such as IL-22 *in vitro* using a novel cell culture system developed for the generation of NK cells. The system, which is based on supportive human mesenchymal stem cells (MSC) indeed led to the generation of ILC3 expressing specific 'markers': NKp44, CD56, and the TF Rorγt as well as a very high secretion of IL-22 after specific stimulation opening possible novel avenues for the clinical use of ILC3 in suitable clinical settings, such as intestinal bowel disease. Finally, a review was written summarizing the current knowledge of human ILCs and their therapeutic potential.

The insights generated within this thesis greatly contributes to a deeper understanding of human neonatal ILCs as well as NK cell development. We generated a staining panel where everyone is able to faithfully identify circulating ILCs. We further generated more insight into neonatal ILC biology, as neonatal ILCs seem to be transcriptionally unique and might share a common developmental pathway with $CD4^+$ T cells with similar *ID3/ID2* ratio > 1. Adding to this, CB ILC1-like exhibited T cell-associated molecules as well as expression of various TRAV/ TRBV genes further indicating a close relationship to T cells and possibly also thymic development. We were also the first to show transcriptional analyses on neonatal ILCs. Furthermore, we found a novel $CD117^-$ NKP, which phenotypically resembles ILC1 - now called ILC1-like cells - able to differentiate into cytotoxic NK cells with a complex KIR repertoire and able to down-regulate NKG2A. This finding indicates that an assumed linear relationship between $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells might not be likely, as $CD56^{bright}$ NK cells were only capable of limited up-regulation of KIR receptors and kept their NKG2A expression. Based on our data, we assume that ILC1-like cells and $CD56^{bright}$ NK cells both contribute to the generation of $CD56^{dim}$ NK cells with ILC1-like cells contributing to mature $CD56^{dim}$ NK cells with a broad KIR repertoire and $CD56^{bright}$ NK cells generating less mature $NKG2A^+$ $CD56^{dim}$ NK cells with limited KIR expression.

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1 Introduction

1.1 The human immune system

Our immune system is vital for our survival in a world full of pathogens. Once the first barrier is broken, most pathogens could rapidly replicate within us. During this rapid replication, pathogens not only multiply in numbers, but can also change their phenotype leading to alterations in their infectioness[1] with potentially subsequent more lethal consequences for the invaded host - that is us. Fortunately, our immune system has evolved to adapt to these changes. Over 1600 genes were identified to be important for our innate and adaptive immune system[2], which are majorly conserved throughout species[3]. That is the reason why we use rodent, canine or other vertebrates as our model organisms. Despite the usefulness of these model systems not all genes and functions can be translated one to one to human immunology. Hence, this study will completely focus on human samples and human literature, if not otherwise indicated in the text.

During foetal development our immune system already starts to develop, which can be seen in cord blood (CB) as well as foetal studies[4]. In addition to our own developing immune system, we are protected from our mother's immune system[5], which will even protect us during the first weeks of life[5] if breast-feeded. Interestingly, a study comparing CB and one to twelve week old infants could already show differences in the immune cell composition[4]. Further a difference between term and pre-term children was detectable[4] suggesting a rapid change within the immune system once the children are born. Infants are exposed to many foreign organisms, which may be harmful, but on the other hand can also shape our immune system e.g. commensal bacteria in the gut[4]. Even though our immune system is relatively immature during infancy, the first line of defence - our innate immune system - has already started developing to protect us[6]. Until adulthood our innate and adaptive immune cells, also called leukocytes, mature more and more to reach optimal performance. However, with increasing age, the functionality of our immune system declines again[6].

Once exposed to pathogens, our immune system follows a distinct reaction path, which is time dependent. First, components of the innate immunity recognise the pathogens and act rapidly. Parallel to the fast-acting innate system, the adaptive immune system starts to produce an optimal defence, but needs more time before targeting the invading pathogens (Figure 1).

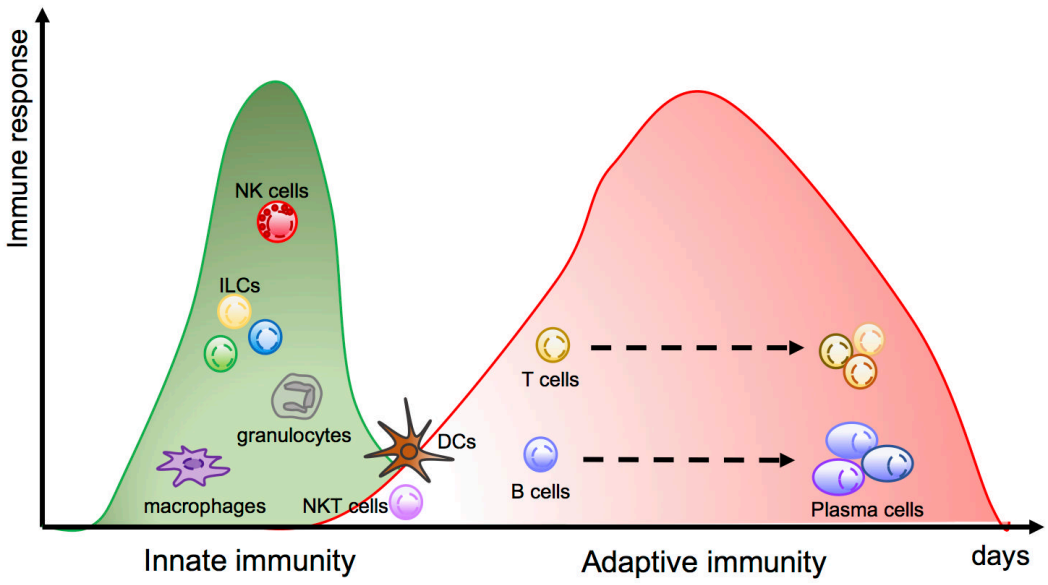

Figure 1: Simplified cell based immune reaction
Granulocytes include neutrophils, basophils, eosinophils. Abbreviations: NK (Natural killer), ILC (innate lymphoid cells), NKT (natural killer T cells), DC (Dendritic cells).

1.1.1 Innate immune system

The innate immune system represents the first line of defence. It consists of different components: our external barriers e.g. the skin, mucous layers, or epithelial layers of the lung or gastrointestinal tract, components within the blood e.g. complement system or other body fluids e.g. saliva. However, once this barrier is penetrated innate immune cells face the invading pathogen. The types of immune cells within the innate immune system are various: macrophages, dendritic cells (DCs)[7], Natural killer (NK) cells (see Chapter 1.2.1), innate lymphoid cells (ILCs) (see Chapter 1.2.3), granulocytes (containing neutrophils, basophils, and eosinophils, and Natural killer T (NKT) cells. Each cell type has its own special way of reacting to pathogens, but all are able to react rapidly once a pathogen was detected without prior activation either by recognising infected body cells or recognising the pathogen itself.
Recognising the pathogen itself, is one essential step during innate immune reaction. Within the innate immune system, a reaction can be triggered once immune cells recognise specific conserved, essential patterns of the pathogens, also known as pathogen-associated molecular pattern (PAMP) via pattern recognition receptors (PRR)[8]. These PAMPs can be extracellular structures on the pathogens or the genetic material e.g. deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) from bacteria, viruses, parasites or helminths[8]. PRR include retinoic acid inducible gene (RIG)-I-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, C-lectin receptors, and toll-like receptors (TLR)[8]. 10 different TLRs have been identified to far[9], which can be found dominantly on antigen presenting cells, such as DCs and macrophages[9]. They are either located extracellularly on the plasma membrane or intracellularly within endosomes[10]. TLRs found on the plasma membrane recognise conserved, extracellular PAMPs on the pathogens surface: TLR11 recognising profilin like molecules[10],

TLR5 recognising Flagellin[10], TLR4 recognising LPS[10], TLR2 alone recognising peptidoglycans and different mannan structures[10], and two heterodimers of TLR2 with TLR1 recognising triacyl lipopeptides[10] or with TLR6 recognising lipoteichoic acid or diacyl lipopeptides[10]. On the other hand, TLRs expressed within endosomes recognise foreign genetic material: TLR3 dominantly recognises double stranded (ds) RNA[10], TLR9 recognises dsDNA with CpG motifs[10], and TLR7 recognises single stranded (ss) RNA[10]. This recognition is not limited to sensing bacteria, but can also detect viruses, protozoa, fungi, parasites and additional self[8]. The sensing of PAMPs by PRRs is essential for initial immune defence.
Instead of sensing PAMPs, NK cells are specialised in recognising infected or abnormal cells via the absence of human leukocyte antigen (HLA)[11]. The function and importance of NK cells, will be discussed in more detail (see Chapter 1.2.1).
Bridging the innate and adaptive immune response are DCs. DCs can either react via recognising PAMPs[8], but can also present antigens to T cells via major histocompatibility complex (MHC) I and II and thus bridging the innate and adaptive immune system[7].

1.1.2 Adaptive immune system

Unlike the innate immune system, which can only react by a recognition of conserved patterns, the adaptive immune system has a much higher frequency of diverse receptors enabling the host to recognise invading pathogens more efficiently. Furthermore, the adaptive immune system has a memory, so it can react faster if the same pathogen invades the second time.

1.1.2.1 Being at the right place at the right time

In parallel to the start of the innate immune systems response, the adaptive immune response starts to react as well. When antigen presenting cells (APCs) within barrier tissues e.g. DCs sense a pathogen, they ingest the pathogen, and start migrating to the secondary lymphoid organs (SLO) e.g. lymph nodes (LN), spleen, and mucosa-associated lymphoid organs (MALT) via the lymphatic system[7]. On the way to the SLOs, DCs process the pathogens to pathogenic fragments, so called antigens, to present them via MHC I and II to $CD8^+$ T lymphocytes or $CD4^+$ T_H lymphocytes respectively[12]. Of note, pathogens or antigens can also reach the SLOs via lymph vessels[13, 14]. Circulating pathogens or antigens can be taken up by immature DCs, who are located at sides of pathogen entry[14]. Unlike DCs or free pathogens who migrate via afferent lymph vessels into SLOs[13], naïve T and B cells migrate into the SLOs via the blood stream through high endothelial venules (HEV) in search for their antigen[13]. This recirculation is highly efficient and is further increased during inflammation[13].
To enter SLOs, leukocytes have to express specific chemokine receptors and adhesion molecules[13]. The interaction of chemokines with chemokine receptors can be seen as road maps guiding the needed cell to their place of use. This process is called leukocyte homing[13]. Of note, immune cells can express several chemokine receptors, integrins, and adhesion molecules. Furthermore, the expression of these receptors varies between different activation stages[14]. Hence, the recruitment into the LN is only one example how the specific recruitment of immune cells into tissues is taking place of several different mechanisms used *in vivo.*

Naïve T and B lymphocytes are carried within the blood stream to screen for antigens to be recognised by their specific receptors: T cell receptor (TCR) or B cell receptor (BCR). The

migration into tissues involves four distinct steps at the HEVs: tethering/ rolling, activation, adhesion, and diapedeses[13].
Naïve leukocytes express L-selectin (CD62L) to mediate tethering and rolling at HEVs, which in return express integrin ligands, such as CD34, GlyCAM-1, or MAdCAM-1 in case of mucosal endothelium. The chemokines CC chemokine ligands (CCL) 21 (secondary lymphoid cytokine, SLC) and CCL19 create a chemokine gradient for the recruitment of circulating leukocytes. CCL21 is dominantly expressed by the high endothelial cells of the HEV[15], while CCL19 is expressed by the lymphatic endothelium, DCs, and intestinal cells of LN-resident cells, which can be transported on HEVs[13, 14]. Among other cell types, naïve T as well as B lymphocytes express the CC chemokine receptor (CCR) 7 enabling them to bind to CCL19 and CCL21[16]. The binding of CCR7 to CCL19/CCL21 starts the activation of integrins[13] preventing leukocyte escape. After activation, naïve leukocytes adhere to the HEV by the integrin leukocyte function-associated antigen 1 (LFA-1) binding to intercellular adhesion molecule (ICAM1)[17]. LFA-1 is a heterodimer formed by CD11a and CD18[17], mediating the arrest of the leukocytes at the HEV[18]. The final step is the transmigration or diapedeses through the HEVs, however the detailed mechanisms are still debated[13]. It has to be noted that this scenario is constantly undergoing changes for example when HEVs start to upregulate additional chemokines during inflammtion[14].

The architecture of SLOs is highly compartmentalised. Within the LN two main histological regions can be determined: cortex and medulla[13]. The cortex can be further divided into the T cell zone, also called paracortex, and B cell zone, harbouring the follicles and - during acute infections - germinal centers[13]. The medulla is a complex network of medullary sinuses, where the efferent lymph vessel drains, separated by medulla cords, which contains plasma cells, memory T cells, and macrophages[13]. Of note, all SLOs have this compartmentalisation, however nomenclature depends on the tissue. Despite the specific names, other cell types like macrophages, DCs, and stromal cells can be found within both regions, which are very important for the structural architecture. Once within the LN, naïve T lymphocytes are recruited into the T cell zone via CCR7 encountering DCs[13, 14]. While B lymphocytes are redirected towards follicles via the CXC chemokine receptor (CXCR) 5 recognising CXC chemokine ligand (CXCL) 13 potentially secreted by follicular stromal cells and/ or follicular dendritic cells (FDC)[14]. Within the follicle, B lymphocytes move in a ‘random-walk’ potentially depending on the CXCL13 gradient[14].

1.1.2.2 Activation of T (and B) lymphocytes

Due to a dense clustering of different cell types within the T cell zone, naïve T lymphocytes can rapidly scan peptide:MHC complexes expressed by DCs via their T cell receptor (TCR)[19]. Two scenarios are possible: either a TCR recognises a peptide:MHC complex, then the former naïve T lymphocytes remains in the T cell zone for activation and maturation or the naïve T cell’s TCR has not found a match, then the naïve T cell re-enters the blood stream looking for the perfect match in another SLO[13]. TCR specificity is already decided very early at week 7-9 during gestation within the developing thymus[20], while complex TCR repertoires can be found within the intestine after 16 weeks of gestation[21]. Importantly, T cell reactivity needs to be balanced: on one hand a diverse TCR repertoire ensures the recognition of various different

antigens, on the other hand autoreactive T lymphocytes must be eliminated. However, in order to be activated, a T lymphocyte needs more than just the one signal from TCR engagement.
Besides the stimulation via a peptide:MHC complex, T lymphocyte need co-stimulation (a second signal) for their survival. The interaction of cluster of differentiation (CD) 28 expressed on T lymphocyte with CD80/ CD86 expressed on APCs, such as DCs but can be also provided by B lymphocytes[22], provide T lymphocyte with the essential survival signal[19]. T lymphocytes who encounter their peptide:MHC complex without stimulation via CD28:CD80/CD86 become unresponsive, also known as anergy[19]. The co-stimulation via CD28 also encourages proliferation, as the engagement via CD28 activates the transcription of interleukin (IL) 2, which promotes T cell proliferation[19]. Lastly, a third signal is needed to determine the functional properties of the activated effector T lymphocytes[23]. This third signal is the co-stimulation of activated T lymphocytes with cytokines during TCR engagement[23]. Several different differentiation phenotypes have been described for T cell: T_H1 via stimulation of IL-12[23], T_H2 via stimulation of IL-4[23], T_h17 via stimulation of TGFβ and IL-6[23], T_H9 via stimulation of TGFβ and IL-4[24], and regulatory T lymphocytes via stimulation of TGFβ alone[23]. Of note, two different types of T cell can be distinguished according to their TCR chains: α:β T lymphocytes and γ:δ T lymphocytes. The latter does not recognise classical peptide:MHC complexes[25]. After a successful immune response, most effector T lymphocytes die, but some T lymphocytes become memory T lymphocytes bound to patrol within the blood and SLOs for a quick reactivation when re-encountering the same antigen[19]. This memory can lead to a life-long protection against a certain type of pathogen.
In contrast to T lymphocytes who develop within the thymus, B lymphocytes develop within the bone marrow (BM). Unlike T lymphocytes whose TCR is membrane bound, B lymphocytes have different isoforms of their BCR: IgA, IgM, IgD, IgG, and IgE, which are either membrane bound or soluble, the latter are then called antibodies. An isoform switch happens via somatic hypermutations within germinal centers[22]. However, when B lymphocytes exit the BM they first express IgM, but start to co-express IgD once matured[25]. B lymphocytes, like T lymphocytes, are activated upon recognition of antigen via their BCR. In contrast to T lymphocytes, B lymphocytes can recognise soluble antigens as well as antigens bound by FDCs[22]. Once activated, B lymphocytes can migrate into the T cell zone via CCR7 by above-mentioned CCL19/CCL21 gradients to present the recognised antigens on MHC II to $CD4^+$ T lymphocytes[22]. Activated B lymphocytes can either become short-lived extrafollicular B lymphocytes secreting the initial wave of antibodies or start clonal expansion within the germinal center[22]. The germinal centre is compartmentalized into a light zone and a dark zone[22]. Within the dark zone, B lymphocytes, also known as centroblasts, divide rapidly and undergo somatic hypermutation[22]. Centroblasts are bound within the dark zone by the chemokine receptor CXCR4[22]. CXCL12 - the ligand of CXCR4 - is produced by dark zone resident reticular lymphocytes[22]. Once the centroblasts successfully completed their somatic hypermutation, CXCR4 is down-regulated and the centroblasts migrate into the light zone[22] and are then called centrocytes. The migration into the light zone is encouraged by FDCs expressing CXCL13[22]. CXCL13 is a ligand of CXCR5 which is expressed on Centrocytes[22]. Centrocytes are positive selected by FDCs and T lymphocyte according to the antigen binding affinity of their BCR[22]. Positive selected B lymphocytes become long-lived IgG secreting plasma cells or memory B cells[22]. These cells are necessary to ensure constant antibody supply to condemn the initial pathogen as well as to enable a rapid defence if re-infected.

1.2 Introducing innate lymphoid cells

Innate lymphoid cells (ILCs) describe a group of cells, which have been identified more than 10 years ago[26]. ILCs, as the name already indicates, belong to the innate immune system. Hence, they are thought to play roles early during the immune response[26]. These cells have a lymphoid morphology[27], but lack rearranged antigen receptors[27], and lineage defining extracellular receptors e.g. CD3 for T lymphocytes[27], CD14 for monocytes/ macrophages[27], and CD19 for the B lymphocytes[27]. In 2013, ILCs were divided into three groups, containing five different cell populations, according to their secreted transcription factor (TF) profile and cytokines: group 1 comprising natural killer (NK) cells and ILC1[26], group 2 comprising ILC2[26], and group 3 comprising ILC3 and lymphoid tissue inducer cells (LTi)[26]. However, in 2018 due to novel insights into ILC biology and development, the idea of three groups was dismissed by leading scientists in the field, instead the five populations are now separate from each other[26]. Despite the separation into five different groups, distinguishing NK cells and ILC1 within literature does not seem to be easy, despite the expression of distinct cell surface receptors on NK cells. Some argue that there is no known stable cell surface receptor to distinguish human NK cells and ILC1s faithfully[28]. Others call their analysed cells `ILC1s`, even though they share more features with NK cells compared to classical ILC1[29-31] and should therefore be treated as NK cells (see Chapter 1.2.3.2). Therefore, it is important to state the kind of cell surface characteristics the cell subset has that is currently analysed within a study and to pay extra attention to the lineage cocktail used.

Due to their described functional capacity, ILCs and NK cells are thought to be the innate counter part of T lymphocytes. In this model, NK cells are the innate counterpart to cytotoxic $CD8^+$ T lymphocytes, while ILC1, ILC2, and ILC3 mirror various types of T-helper (T_H) cells[26, 32, 33], whereas LTis do not correspond to a T cell subset (Figure 2).

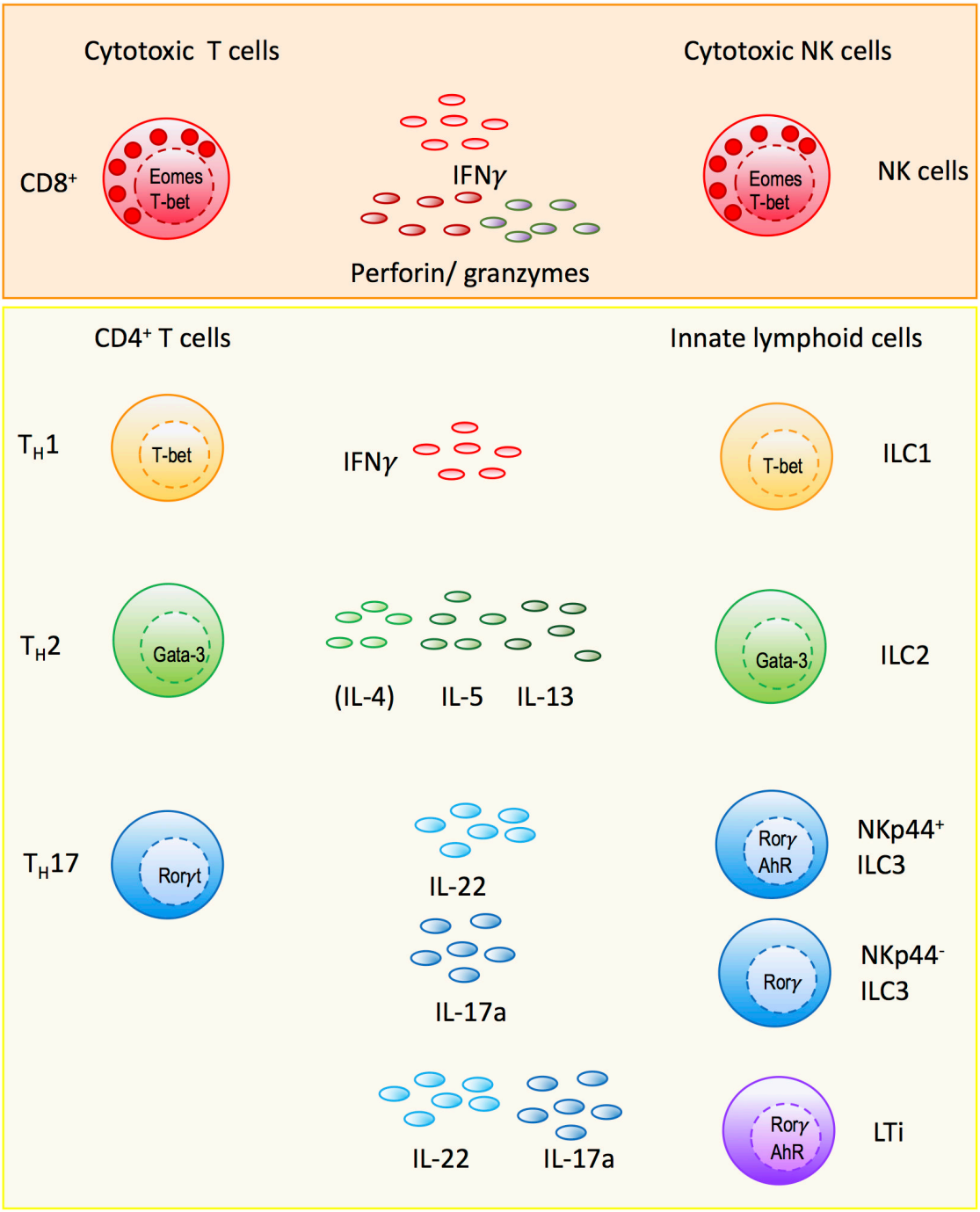

Figure 2: Simplified view of ILC cytokines and transcription factors mirroring T cells

Figure adapted from Bernink *et al.* (2013)[34], Mjösberg *et al.* (2011)[35], Hoorweg *et al.* (2012)[36], Vivier *et al.* 2018[26], Spits *et al.* (2013)[32], and Eberl et al (2015)[37]. Abbreviations: ILC (innate lymphoid cells), NK (natural killer), IL (interleukin), T_H (T-helper), CD (Cluster of differentiation).

ILCs have been described to be important in many different areas of the immune system. Being first only recognised as the innate counterparts of T cells, they became more and more appreciated to play vital roles within mucosal barriers and tissue homeostasis[26]. ILCs might also become more attractive subjects in clinical terms, as circulating ILCs seem to be resistant

to chemotherapy[38] and their frequencies might be early indicators to predict septic shocks[39]. Their potential clinical relevance is one of the reasons why ILC research is still a very active field at the moment. However, ILC research also brings certain challenges: not every type of ILC is found within the same organ, ILCs might not exhibit the same phenotype in different tissues, no uniform nomenclature as well as staining panels have been agreed upon, despite several attempts[32, 40, 41], and finally, to make the life of an ILC researcher even more difficult, these cells are very rare in the circulation as well as in most tissues[42]. Of note, within this thesis the term ILC will only refer to ILC1, ILC2, and ILC3, but not NK cells, as outlined below. Furthermore, this thesis will only refer to human ILCs and NK cells, if not otherwise stated.

1.2.1 Natural killer (NK) cells

The two main human NK cell subsets are characterized by their expression level of CD56: $CD56^{bright}$ NK cells expressing high levels of CD56 and $CD56^{dim}$ NK cells expressing lower levels of CD56. NK cells can be further classified as being negative for classical lineage receptors such as CD3 for T cells, CD14 for monocytes, and CD19 for B cells like ILCs. Even though different phenotypes for NK cells have been identified within different organs[43], NK cells can be faithfully identified due to the expression of distinct receptors on their cell surface, such as CD56 and NKG2A - within the liver, SLOs, BM, lung, kidney, and gut - or other prominent NK cell receptors - on uterine NK cells[43] - indicating that tissue environment influences NK cell phenotypes. Even though, NK cells are vitally important for host defence, human NK cell development still remains to be not fully understood (see Chapter 1.4.1). The two subsets can be discriminated upon their different phenotypic appearance and functionality.

1.2.1.1 $CD56^{bright}$ NK cells

$CD56^{bright}$ NK cells exert more regulatory functions, as they are very potent producers of IFNγ[43], but have hardly any cytolytic activity[43]. They are thought to be the main NK cell subset to have modulatory capacity to interact with DCs and T cells[44], as human LN $CD56^{bright}$ NK cells secrete high amounts IFNγ after stimulation with IL-2 produced by activated T cells[45] and can be activated by DCs[46, 47]. In line with their cytokine producing phenotype, $CD56^{bright}$ NK cells do not express CD16 needed for the ADCC or killer cell immunoglobulin-like receptors (KIR), but faithfully express the heterodimer CD94:NKG2A[48], belonging to the C-lectin family, recognising the non-classical MHC class IB molecules human leukocyte antigen (HLA) HLA-E[49]. The process of NK cell activation will be explained in the $CD56^{dim}$ NK cell section.

1.2.1.2 $CD56^{dim}$ NK cells

$CD56^{dim}$ NK cells are dominantly cytotoxic and can vary in their CD16, NKG2A, and KIR expression[43]. Compared to $CD56^{bright}$ NK cells, which faithfully express the heterodimer CD94:NKG2A[48], $CD56^{dim}$ NK cell show various different phenotypes. $CD56^{dim}$ NK cells can express the heterodimer CD94:NKG2A alone or with different numbers of KIR receptors, but can also be double negative for NKG2A or KIR expression[50]. It could also be shown that $CD56^{dim}$ NK cells can undergo a further terminal differentiation step into mature NK cells, which down-regulate NKG2A from their cell surface, but maintain KIR expression with up-regulation of CD57, becoming mature $NKG2A^{-}KIR^{+}CD57^{+}$ NK cells[51]. Of note, a special $CD56^{dim}$ NK cell subset expresses the heterodimer CD94:NKG2C. $NKG2C^{+}$ NK cells are mainly seen after an acute infection of human cytomegalovirus (HCMV)[52].

CD56^{dim} NK cells use different molecules to distinguish between foreign and self. CD16, also known as Fcγ receptor III (FcγRIII), is expressed on CD56^{dim} NK cells to recognise the Fc part of Immunoglobulin G (IgG) antibodies, which have been produced by plasma cells specifically against a pathogenic fragment/ pathogen (see Chapter 1.1.2.2). The specific IgGs coat the extracellular surface of the target cells or pathogens and are then recognised via CD16, which activates an intracellular signal cascade via CD3ζ or FcεRIγ chains within the CD56^{dim} NK cells[53]. Once recognised, NK cells kill their target cells very effectively[43]. This process is called antibody dependent cell-mediated cytotoxicity (ADCC).
KIR and NKG2s are HLA class I-specific receptors enabling NK cells to recognise the absence of MHC-class I molecules[54] on virus infected, damaged or malignant cells, as all healthy body cells express MHC-class I molecules, except erythrocytes. As the cytotoxic ability of CD56^{dim} NK cells is very potent and IFNγ secreted by both - CD56^{bright} NK and CD56^{dim} NK cells - is inflammatory, the process of activating NK cells needs to be tightly regulated. NKG2s/KIRs consist of inhibitory and activating receptors and generally activation of the inhibitory receptors overrules the activation of the activating receptors. As a rule of thumb, if a NK cells is activated and simultaneously inhibited due to the expression of self-MHC class I on the recognised cell, the NK cell will be inactivated. Similarly, without the engagement of an inhibitory receptor, the NK cell is activated - called 'missing-self hypothesis - and starts its cytotoxic activity or cytokine secretion[55]. Importantly, CD16 and NKG2D can override the effect of NKG2A and inhibitory KIRs, so the NK cells get activated despite an inhibitory signal[56].
KIR^{+} CD56^{dim} NK cells can recognise HLA-A/B/C/G depending on the specific KIR expressed on the NK cell[50], while the heterodimer CD94:NKG2A[48] recognises the non-classical MHC class IB molecule HLA-E[49]. Depending on the recognised HLA class I molecule, KIR^{+} CD56^{dim} NK cells can either be 'licensed', or remain 'unlicensed'. Which KIR is licensed depends on the expressed HLA class I genotype of an individual. HLA-C has two alleles: HLA-C1 and C2, which can be expressed alone (either having the genotype C1/C1 or C2/C2) or simultaneously (genotype C1/C2)[50]. Both HLA-Cs serve as self-ligand and therefore can inhibit NK cells. Independent of the genetic make-up of an individual towards either HLA-C allele, KIR genes are not uniformly expressed but are polymorphically encoded in the individuals germline[57] with very complex underlying genetics[50]. This results in a very broad repertoire of KIR^{+} CD56^{dim} NK cells with selectively 'licensed' CD56^{dim} NK cells[50]. CD56^{dim} NK cell expressing KIR2DL2/L3 are licensed when recognising self HLA-C1[58, 59], while CD56^{dim} NK cells expressing KIR2DL1 are licensed by recognition of self HLA-C2[60]. Hence, CD56^{dim} NK cells can either be licensed for one or both HLA-Cs, as the term refers to the specific recognition of self HLA-C1 or C2 by their specific KIR[61]. Of note, KIR3DL1 expressing CD56^{dim} NK cells can be licensed when recognising Bw4[62], which is expressed on HLA-B and some HLA-A alleles. Studies suggest `licensed` CD56^{dim} NK cells to be functionally more competent compared to 'unlicensed' NK cells, as they have recognised their specific self HLA-C molecule[61], nevertheless the whole process of licensing is still elusive and still the subject of intensive research.

Two pathways to kill their target cells have been described for CD56^{dim} NK cells: the first uses cytotoxic granules and the second uses the tumour necrosis factor (TNF) receptor family or associates. The indirect induction of target cells apoptosis is mediated by perforin and

granzymes. Once a target cells is recognised, a lytic synapse is formed between NK cell and target cell ensuring tight adhesion via the LFA1 (CD11a/CD18) or MAC1 (CD11b/CD18) complex[63]. This lytic synapse formation is a very complex system[63] and will not further be discusses in detail. Within their cytosol NK cell have cytotoxic lysosomes containing the enzymes perforin and granzyme B. To prevent a self-inflicted killing, a low pH value inactivates both perforin and granzyme within the lysosomes[64]. Once perforin and granzyme are released within the lytic synapse, the inner membrane of the lysosomes is coated with CD107a, also a lysosomal protein, to prevent a perforin and granzyme induced killing of the NK cell itself. NK cells further secrete Cathepsin B and Serpin, which destroy the lytic activity of perforin and granzyme[65, 66]. Perforin enables the granzyme to enter the target cells, however the exact mechanism is still a matter of debate. Several different granzymes are known, but not all of them are well characterised. $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells differ within their granzyme expression: while $CD56^{bright}$ NK cells dominantly expresses Granzyme K[67], $CD56^{dim}$ NK cells express Granzyme A, B, and H, with Granzyme B being the most studied one. Granzyme B is a serine-protease cleaving caspase 3, which initiates apoptosis and at the same time disrupts mitochondrial integrity[68]. Direct apoptosis on the other hand can be initiated with FAS belonging to the TNF superfamily recognising FAS ligand (FASL) on the target cells or by up-regulation of TNF-related apoptosis inducing ligand (TRAIL) which is recognised by TRAIL receptor on the target cells, directly inducing apoptosis[69].

1.2.1.3 NK cell function

NK cells are essential for the innate immune system. The main function of NK cells is the ability to kill virus infected, damaged or malignant cells when they have down-regulated major histocompatibility complex (MHC) class I molecules[54]. On the other hand, NK cells can also interact with various immune cells e.g. DCs and T cell. Therefore, NK cells also have important immune modulatory roles[43]. Due to their special abilities, NK cells are very important for therapeutic purposes[70, 71].

The importance of NK cells can be especially seen during the reconstitution of the immune system after hematopoietic stem cell transplantation (HSCT). HSCT is a potential curative treatment in a subset of leukemic patients, who are non-responsive to chemotherapy and have frequent relapses[72]. NK cells, especially $CD56^{bright}$ NK cells, have been described to be one of the first cell populations to reconstitute within the patient[73], indicating their importance. Of note, the frequency and quality of reconstitution within the first month after HSCT significantly influences morbidity and mortality of the patients[74]. A potent graft-versus-leukaemia (GVL) effect has been observed from effector cells within the stem cell graft e.g. T cells and NK cells. However, due to recognition of allogeneic HLA class I molecules (direct allorecognition) as well as recognition of alloreactive HLA class I/peptide combinations (indirect allorecognition), alloreactive T cells are strong mediators of graft-versus-host disease (GvHD), while NK cells do not promote GvHD partly due to their expression of inhibitory receptors broadly recognising HLA class I molecules[75]. Furthermore, patients with GvHD showed delayed NK cell reconstitution[76]. Interestingly, a novel NK cell subset was observed after HSCT showing features of both NK cell subsets. These cells are called $CD56^{Intermediate}$, as they are $CD56^{bright}$, but express moderate levels of CD16 and have effector functions[77]. However, a prolonged peak of these cells was associated with chronic GvHD[76] potentially indicating an important role early

after HSCT, but not later on. The importance of NK cells for cancer therapy can also be seen in the rapid dynamic field of individually engineered cancer therapy by the usage of NK cells with chimeric antigen receptors (CAR). CARs consist of a small engineered fragment, from the variable region of an antibody, recognising a specific epitope, a transmembrane domain, and an intracellular endodomain often containing two or more stimulatory domains in tandem from molecules such as CD28, CD137, and CD3ζ[78, 79]. With the help of the specific epitope recognition, the CAR-NK cells can specifically kill the epitope expression target cells. Early this year, a first clinical trial using allogeneic anti-CD19 CAR NK cells against $CD19^+$ tumours showed impressing clinical benefits without having major cytotoxic effects[79]. The respective CAR NK cells persisted within the patients for up to 12 months, suggesting that CAR NK cells may provide a novel route for allogeneic treatment of cancer patients.

1.2.2 Lymphoid Tissue inducer cells (LTi)

In the original nomenclature LTi were part of group 3 ILCs (with ILC3) but were later separated based on a.) functional differences: LTis are essential for early foetal development, especially the formation of the SLO[80], while circulating and tissue ILC3 are found within CB, periphery post-birth, and many different tissues[36, 42, 81, 82] and b). developmental differences:

LTis were first identified within neonatal murine LNs as $CD45^+CD3^-CD4^+CD44^+Thy\text{-}1^+B220^-$ $MAC\text{-}1^-$ cells[83] and are now typically classified as $lin^-CD127^+CD117^+Ror\gamma t^+\alpha_4\beta_5^+CD4^{+/-}$[80]. Human LTis have a similar phenotype as murine LTis, however they lack CD4, while expressing intracellular adhesion molecule 1 (ICAM-1, CD54), CD7[84], and as recently identified, also neuropilin-1 (NRP-1)[85]. LTis migrate from the foetal liver into the blood stream to potential sides of LN anlagen and peyer's patches (PP) development[86]. LTis are recruited to these sides by mesenchymal stem cells (MSCs) via the up-regulation of mucosal vascular addressing cell adhesion molecule 1 (MadCAM1) at HEVs[87]. Once within the LN anlagen, LTis express lymphotoxins (LTs), which are members of the TNF family[80, 84]. LN development depends on the secretion of Lymphotoxins (LTs) by LTis. It has been observed that different types of LN depend on different LTs. For example, LT-α_3, a soluble homotrimer of the LT- α chain, supports the development of cervical and mesenteric LNs as well as potentially lumbar and sacral LNs, which has been identified within LT-α-deficient mice[88]. On the other hand, LTβ (a heterodimer of LT- α_2 β_1) supports the development of all other LNs including PP and germinal centers[88]. LT-α/β receptor expressing stromal cells respond to LT secretion by LTis by further up-regulation of adhesion molecule expression, which leads to the formation of LTi clusters[80]. Within these clusters, LTis secrete IL-7 and RANK-ligand to initiate autocrine and paracrine cluster survival, proliferation, and differentiation[89]. Furthermore, activated LTi clusters express LN specific chemokines, such as CXCL13, CCL20, and CCL19 to recruit T cells, B cells, and DCs to the LN[90] (see Chapter 1.1.2). Of note, LTis can also secrete IL-17 and IL-22, like ILC3, however their biological impact on the development of LN is currently unknown[80].

1.2.3 Innate lymphoid cells (ILCs)

The name ILCs for a group of cells within the innate immune system makes sense at first, but becomes confusing when this only refers to a specific group of cells. While other lymphoid cells belonging in the innate immune system, such as NK cells, do not belong into this group.

Nevertheless, it is now commonly accepted that ILCs comprise three different subsets: ILC1, ILC2, and ILC3. Historically, the three ILC subsets were grouped together due to the common lack of cell surface molecules associated with the erythroid and myeloid lineage and the absence of antigen-specific T- and B-cell receptors. Instead, the exhibit shared expression of CD127 (IL7R) and killer cell lectin-like receptor subfamily B (KLRB1; also known as CD161)[33]. Another feature of all ILCs was the absence of RAG enzymes responsible for rearranging antigen receptors[33]. Adding to this might be the fact that their described functionality constitutes an innate functional counterpart to the three adaptive $CD4^+$ T cell subsets as illustrated in Figure 2. This classification was also done with invariant natural killer T cells (NKT), which are classified into iNKT1, iNKT2, and iNKT17 corresponding to T_H1, T_H2, and T_H17, respectively[91]. Two publications recently showed that human ILCs and T_H cells share important regulatory mechanisms[92, 93] suggesting a developmental relationship towards each other.
Looking at murine studies, the classification of all three ILC subsets into one common group appears to be more straightforward. Murine ILCs share the dependence of the TF ID2 and PLZF during their development[26]. Even though PLZF was also observed to be expressed within tissue ILCs[94], it is questionable, if the described developmental murine ILC pathway is transferrable to human. Some studies already exist comparing the transcriptomic data of all the ILC subsets to each other. In line with the commonly accepted gating strategy, CD161 and CD127 were expressed on all tissue und PB ILCs. When comparing tissue ILCs a shared TF signature was observed including *RORA* and PLZF (*ZBTB16)*[94], while in PB most genes were mitochondrial and ribosomal genes[95] questioning if ILC1, ILC2, and ILC3 should be classified within the same group.
Interestingly, all three -murine and human- ILC subsets share the absence of rearranged recognising receptors on their cell surface, which makes ILCs the only lymphocyte population without antigen or self-recognition capacity (T cell recognise via TCR, B cell recognise via BCR, NK cells recognise via NKG2s and KIR, and iNKT cells recognise via CD1d). Potentially in the future a more specific name might be useful for ILCs such as innate lymphoid cells without antigen receptors (ILCwar). Despite their shared name, the three ILC subsets have been described to have unique functions.

1.2.3.1 ILC1

Human ILC1 have been originally described within healthy tonsils and the gastrointestinal tract[34], as lineage $(lin)^-$ $CD127^+CD117^-CRTH2^-NKp44^-$ cells expressing the TF T-bet and secreting IFNγ after interleukin (IL)-12 and IL-18 stimulation[34]. These tissue ILC1 further expressed CD161[34], which has been described to be expressed on all ILC subsets as well as both NK cell subsets[32]. Due to the absence of unique cell surface receptor expression, human ILC1 are the least well defined ILC subset.
Apart from the initial characterisation[34], human ILC1s were described to express dominantly T-cell associated characteristics[94, 96, 97]. Several different studies observed the expression of CD5[94, 96, 98], the co-stimulatory molecule CD28[94, 96], CD6[94], and intracellular CD3ε[94, 96]. Of note, CD28 expression was observed within all three ILC subsets[96], however functional relevance of CD28 on ILCs remains to be elusive. Furthermore, one group showed a separation of PB ILC1 according to their respective CD4 and CD8 expression: $CD4^+$ILC1, $CD8^+$ILC1, and double negative (DN)ILC1s[96]. Due to the lack of a clear-cut phenotypic appearance of ILC1 cells some publications questioned the existence of ILC1s or speculated that they are a result

of T cell contaminations[99], which lead to the inclusion of CD5 within the lineage cocktail by a recent publication[100]. Other studies also reported that they did not proceed to analyse ILC1s within their study due to infrequent existence of the originally , as 'CD161^{+}', described cells[101]. Due to their location within tonsils and the gastrointestinal tract as well as their potential to mirror a T_H1 response[34], ILC1 were described to be the corresponding innate counterpart. However, due to the less than clear-cut phenotypic description separating these cells from NK cells or T cells, it is difficult to anticipate what the function of these cells really are. Nevertheless, increased frequencies of the originally defined ILC1[34] were found in the inflamed terminal ileum of patients with Crohn`s disease[34, 102] and in patients with ulcerative colitis[103] suggesting to aggravate the diseases by secretion of IFNγ[34]. Hence, evidence suggests a role of ILC1 in the pathogenesis of bowl diseases.

1.2.3.2 ILC1 with a NK cell-like phenotype

A very important point while studying ILC1s, is the ability to faithfully distinguish ILC1 and NK cells. As Eomes is essential for NK cell maturation[104], but not ILC1, the expression of Eomes was originally used to distinctively separate ILC1s to NK cells[32]. Nevertheless, Eomes expression has been described in peripheral blood (PB) ILC1s[95, 96] erasing a black and white picture of the transcriptional identity. Adding to this, two different ILC1 subsets have been described within literature, which, based on their expression of NK cell receptors, rather belong to the NK cell lineage than to ILC1. Thus, human ILC1s are a very challenging population to study and a concise nomenclature is needed to faithfully separate them from either T or NK cells.

A CD103^{+}ILC1 intraepithelial subset, also known as intraepithelial ILC1s[30] or ILC1a[29] has been described within the human intestine. Despite the original description of ILC1 as lin^{-} CD127^{+}CRTH2^{-}CD117^{-}NKp44^{-} within the tonsils and intestine[34], CD103^{+}ILC1s were originally defined as CD56^{+}NKp44^{+}CD103^{+}[30] and called ILC1. Both described ILC1 subsets express the TF T-bet, but not Eomes, and secrete IFNγ[30, 34]. However, CD103^{+}ILC1 can be regarded as NK cells, as they express CD94[105] on the cell surface and have intracellular granzymes[29]. Another indicator for this assumption is the clustering with NK cells in a principle component analyses (PCA)[29, 106]. Notably, CD103 - also known as human mucosal lymphocyte-1 antigen - constitutes a receptor for E-Cadherin (as heterodimer with integrin beta 7) and is a classical marker of intraepithelial lymphocytes (IEL). Importantly, CD103 expression has been described on various T cell and NK cell subsets within mucosa associated lymphoid tissues (MALT)[43] as well as the lung[107], and thus cannot be regarded as NK exclusion `marker'.

A CD56^{+} ILC1 subset has been described in PB of healthy blood donors as well as in acute myeloid leukaemia (AML)[108] and metastatic colorectal carcinoma[31] patients. These findings need to be treated carefully, as these studies used only CD16 within their lineage cocktail to exclude NK cells. Due to shedding by ADAM metalloproteases, surface expression of CD16 is eliminated from the cell surface quickly upon activation. Furthermore, as described above CD16 is not expressed on CD56^{bright} NK cells and only expressed on a subset of CD56^{dim} NK cells. A contamination with CD56^{bright} NK cells seems to be a very likely explanation for these findings, as CD56^{bright} NK cells do also express CD127[109]. This can be avoided with a careful gating strategy. Commonly, leading publications within the ILC field use CD94 for NK cells

either within the lineage cocktail or separately and then gate on lin$^-$CD127$^+$ cells for all three ILC subsets[40]. Hence, a contamination of CD56$^+$CD127$^+$ without the usage of CD94 in the lineage cocktail is a likely scenario.

1.2.3.3 ILC2

ILC2 were originally defined as lin$^-$CD127$^+$CD117$^{+/-}$CRTH2$^+$ in PB and nasal pulps[35]. They express CD161, the TF Gata3, and secrete the interleukins IL-5 as well as IL-13 upon stimulation with IL-2 and either IL-25[110] or IL-33 or both[35]. Stimulation via IL-33 on murine ILC2 resulted in an induction of the enzyme tryptophan hydrolase 1 promoting ILC2 functionality[111]. They were further divided according to their CD117 expression, where the CD117$^{-/low}$ population can secrete IFNγ or IL-17A[112], whereas the CD117$^{+/high}$ is mainly associated with lung inflammation and asthma[112-115]. Due to their distinct expression of CRTH2, ILC2 are easily identified. However, CRTH2 expression is down-regulated after T_H2 cell activation and thus potentially also in ILC2[116].

Due to their cytokine profile, ILC2s have been associated with helminth infections, allergic reactions[117], but also tissue repair[118]. Despite their presence within nasal pulps and PB, ILC2s have been identified in various different tissues e.g. lung[113, 119], intestine[120], brain[121], and the skin[122]. Over the last few years, ILC2s have been the focus of attention especially in murine studies[119, 120, 123-126]. Reasons for this enhanced interest in this particular cell population might be the constant increase of allergic burden within the population, especially within children[127], and the fact that ILC2s have a unique phenotypic identity. Recent studies not only gave more insight into ILC2 biology, but also show how interactive the immune system is. Murine ILC2s were described to interact with $\gamma\delta$T cells[128], were described to potently suppress a T_H1 response during fungal infection[126], were shown to be responsive to neuropeptides[120], and might aggravate brain ageing[121]. Furthermore, advances in allergy research have been made as a 'microRNA 146a has been identified to negatively regulate IL-33 in activated murine ILC2'[129] and the receptor activator of NF-κB (RANK) has been identified on human ILC2 to induce inflammation in chronic rhinosinusitis[130], which both might be potential targets for allergic treatment. Recent evidence further suggests that murine ILC2 are trained pre-birth (neonatal period) with endogenous IL-33 leading to aggravated ILC2 responses after a second exposure with IL-33 in adulthood compared to ILC2 derived from IL33-deficient mice[131], if this is also true within human remains to be elusive. Adding to this, a bromodomain and extra-terminal (BET) inhibitor, called iBET151, was identified to reduce cytokine secretion within human ILC2s *in vitro* and experimental murine studies[132]. Of note, unconventional human ILC2 based on the cell surface markers CD30, TNFR2, and ICOS have recently be described to secrete IL-13 and IL-5, but phenotypically lack CRTH2 and/ or CD117 expression[133]. As ILCs are thought to be very plastic (see Chapter 1.3) it is questionable if these cells are indeed ILC2 or if other cells produce ILC2-associated cytokines.

1.2.3.4 ILC3

Among the three ILC types, ILC3s were the first to be identified, however they were not originally described as ILC3, but as IL-22-producing NK cells expressing CD56 and NKp44 within mucosal associated lymphoid tissue (MALT)[134]. One year later the same group showed functional plasticity of these cells, as the presence of IL-2 encouraged the production of IFNγ,

while cultivation with IL1β resulted in IL-22 production[135]. Unlike NK cells, the IL-22-producing CD56$^+$NKp44$^+$ cells expressed high levels of the transcription factor (TF) *RORC,* similar to the previously described LTis[136]. After analysing and comparing LTi and CD56$^+$NKp44$^+$ cells to conventional NK (cNK) cells, it was concluded that LTi and CD56$^+$NKp44$^+$ cells belong to the same cell lineage and do not belong to conventional NK cells[136]. It was only later found that murine LTi and ILCs have a separate precursor termed LTiP and CHILP, respectively[26]. Interestingly, ILC3 are also generated within *in vitro* NK cell cultures generating NK cells from CD34$^+$ hematopoietic stem cells[137, 138] creating a developmental link between these two cell populations. The challenge for investigators remains to distinguish ILC3 and stage 3 of the tonsillar NK cell development (see Chapter 1.4.1), as both express CD117 and CD56[137].
Recently it has become obvious that ILC3 have to be divided into two groups: tissue-resident ILC3s and peripheral ILC3 cells, also called ILC3-like cells or ILC precursors (ILCP)[81]. To stress the phenotype of these circulating CD117$^+$ cells, within this study the term ILC3-like cells will be used. Tissue ILC3 are able to secrete either IL-17A or IL-22 after stimulation[36], while peripheral ILC3-like cells were shown to contain an immature precursor population[81, 100].

1.2.3.4.1 Tissue ILC3

Human ILC3 are defined as lin$^-$CD94$^-$CD56$^{+/-}$CD127$^+$CD117$^+$CRTH2$^-$Rorγt$^+$AhR^{+}[26]. During the past years, two ILC3 subsets have been described. These subsets were distinguished according to their NKp44 expression and their different tissue location. NKp44$^-$ILC3s were identified within foetal LN[36] and amniotic fluid[139] secreting IL-17A. NKp44$^+$ILC3 on the other hand were originally found within tonsils, but also within the gastrointestinal tract to express IL-22[36, 94]. Even though ILC3 are characterised by their respective TF expression of RORC, which is shared with T_H17 cells - the assumed adaptive counterpart (Figure 2) - murine intestinal ILC3s do not seem to be directly dependent on the TF Rorγt to exhibit functionality. A study using a Rorγt inhibitor effectively showed reduced functionality within T_H17 cells, but not in ILC3[140]. One explanation might be that *RORC* is essential for ILC3/ T_H17 development, as patients deficient for *RORC* failed to exhibit any ILC3/ T_H17 effector function[141]. Instead, mature (murine) ILC3 might be more dependent on the TFs aryl hydrocarbon receptor (AHR) and STAT3 for functionality, as AhR-deficient mice showed increased apoptosis and decreased IL-22 secretion within Rorγt$^+$ILC3[142], while STAT3 expression was essential for IL-22 secretion[143]. ILC3 are dominantly described to be important for the maintenance of barrier tissue homeostasis including fast activation upon tissue damage and were described to play important roles within the gastrointestinal tract, also by interacting with commensal bacteria and processed food[33]. Although ILC3 have been described to secrete various different cytokines, IL-22 is the hallmark of ILC3 function. Especially IL-22 is described to be beneficial for tissue homeostasis and maintenance of balance, as its corresponding receptor IL22RA1-IL10RB (IL-22R) is dominantly expressed on non-hematopoietic lineages, especially on intestinal stem cells[144, 145]. Nevertheless, both cytokines (IL-22 and IL-17A) were described to maintain immune barrier homeostasis, as both cytokines predominantly act on epithelial cells[146].

IL-17A production by ILC3 is mainly induced by inflammatory conditions e.g. secretion of IL-1β and IL-23 by myeloid cells or pathogenic threat e.g. fungal or bacterial infections. IL-17A

production results in enhanced anti-microbial peptide and epithelial tight junction synthesis[147] as well as the attraction of myeloid cells[148]. Despite the role in tissue homeostasis, elevated IL-17A levels trigger inflammation[146]. Apart from innate defence mechanisms, elevated IL-17A levels were described to play a role in autoinflammatory diseases[146] e.g. in inflammatory bowel disease[149]. A study suggests NKp44$^-$ILC3 along with ILC1 to be able to aggravate Crohn`s disease by secretion of IL-17A[149].

IL-22 secretion of murine ILC3 seem to be tightly regulated within the gastrointestinal system. Sensing of either commensal or pathogenic microbes by microbial metabolic-sensing receptors e.g. Ffar2 or AhR ligands, enhances proliferation and IL-22 production of murine ILC3 resulting in maintenance of mucus-associated proteins and anti-microbial peptides production by epithelial cells leading to tissue homeostasis[150-154]. Of note, AhR ligands can either be food derived by cruciferous vegetables or microbial origins[155]. Maintaining tissue homeostasis also involves the recruitment of T_H17 cells by ILC3 for mucosal defense[150]. Mice deficient for these receptors or depleted of microbes showed increased susceptibility to pathogens causing tissue injury[150, 151, 153] suggesting the importance of ILC3 for gastrointestinal balance. The secretion of IL-22 was also shown to be influenced by Glial cells via neuroregulators[156]. Recently, a novel human ILC3 was described expressing the ectoenzymes CD39 and CD73 and were hence called: ecto$^+$ILC3[157]. Ecto$^+$ILC3 were described to sense danger-associated molecular patterns e.g. extracellular adenosine triphosphate and responding by secretion of IL-22[157]. The importance of ILC3 within the gastrointestinal tract was further recently shown in patients with intestinal transplantations. Here, the frequency of IL-22-producing ILC3 was significantly decreased in patients with graft rejections, while within patients with successful transplantation ILC3 frequency significantly increased over the first 6 month post-transplant leading to the conclusion that the absence of early seeded ILC3 (2-4 weeks after transplantation) could be a biomarker for transplant rejections[158]. This is in direct contrast to murine data, which showed that the recipient ILC3 are therapy resistant and remained[159].
Interestingly, some of our daily nutrition's seem to lead to controversial ILC3 activation data. Food uptake has been described to activate a group of enteric neurons, which express the vasoactive intestinal peptide (VIP). ILC3 located within the lamina propria were found to express the corresponding VIP receptor type 2 (VIPR2) and were located in close proximity[160]. While the sensing of microbes leads to IL-22 secretion triggering anti-microbial peptide formation, two different scenarios have been published concerning the VIPR2$^+$ILC3 engagement with VIP-expressing neurons after food uptake. In one study VIPR2-VIP engagement resulted in decreased IL-22 production by ILC3 leading to a subsequent decrease in anti-microbial peptide production with an increase of lipid binding receptors for food uptake[160], while another study showed an increase of IL-22 producing ILC3[161]. More studies are needed to unravel these ambiguous findings[162]. Of note, Vitamin D was shown to decrease murine ILC3 function[163]. However, it still remains elusive if ILC3 can sense via all these different mechanisms by themselves. Recently, mononuclear phagocytes (MNPs) were identified sensing microbes with correspondent release of IL-1β and IL-23[153]. Both these cytokines were identified very early on to be important stimulatory cytokines for ILC3[134].

IL-22-secreting ILC3 are normally found within mucosal barrier tissues, but not within the circulation of healthy individuals[81]. As stated above, two functional types of ILC3 have been

described: one secreting IL-22 and the other IL-17A[36]. In line with different cytokine secretion, they show different cell surface expressions of NKp44. While IL-22-secreting ILC3 express NKp44, IL-17A-secreting ILC3 do not express NKp44. So far, to the author`s knowledge, no NKp44$^-$ ILC3 have been described to secrete IL-22.

After hematopoietic stem cell transplantation (HSCT) donor-derived NKp44$^+$ILC3 were found within PB of leukemic patients, which were not seen prior HSCT[164]. HSCT could be a curative cure for leukemic patients non-responsive to chemotherapy with frequent relapses[72]. Unfortunately, an unwanted side effect of HSCT is GvHD. GvHD is caused by alloreactive donor T cells attacking the recipients' organs, especially the liver, skin, and gastrointestinal tract, causing high morbidity and mortality[72]. In HSCT-treated patients, higher frequencies of NKp44$^+$ILC3 were correlated with a reduced susceptibility to GvHD[164] making NKp44$^+$ILC3 an interesting target for future research. Of note, ecto$^+$ILC3 were depleted after GvHD[157] suggesting that the donor can replenish NKp44$^+$ILC3, but not ecto$^+$ILC3. Even though no IL-22 was measured in this study, it was hypothesised that these circulating NKp44$^+$ILC3 were able to secrete IL-22 protecting from aggravated GvHD, as they expressed gastrointestinal and skin homing chemokine receptors[164]. In accordance, the secretion of IL-22 by murine ILC3s has been described to protect murine intestinal stem cells during experimental GvHD[159]. However, a new study suggests that murine intestinal stem cell repair is dependent on ILC3-driven signals, but not on the secretion of IL22[165]. Instead, ILC3 increase the Hippo-Yap1 signalling pathway within the intestinal stem cells preventing pathological damage[165]. During experimental GvHD, the thymus was also described to be a target of inflammation, here IL-22-secreting murine ILC3 were described to protect the thymus[166] and be essential for thymic regeneration[167]. It is known that the thymus regenerates during ageing, in parallel murine ILC3 were shown to significantly decline from pre-birth to adulthood, while ILC2 remained stable and stayed protective[168]. Of note, within the murine thymus only ILC2 and ILC3 were found, but not ILC1[166, 169]. These findings have not been recapitulated in humans yet, but they suggest that human NKp44$^+$ILC3 are indeed important modulators to reduce the risk and consequences of GvHD in leukemic patients. If this prevention is due to the secretion of IL-22 or another mechanism remains to be elusive.

1.2.3.4.2 Peripheral ILC3-like cells

Within the periphery, ILC3 neither express NKp44, the TF Rorγt nor secreted IL-22 or IL-17A[81]. Instead, when co-culturing peripheral ILC3 on the stromal cell line OP9, these cells were able to differentiate into all three ILC subsets as well as NK cells[81]. Leading to a model where ILCs are enabled to migrate into the tissues for further maturation under the local environment[170], which was called 'ILC-poiesis'[170]. Thus, peripheral ILC3 are considered as `ILC3-like` cells. It is important to stress that this study did not further consider other possible functions of these cells, but limited their hypothesis to the secretion of IL-22 and IL-17A.

When further divided according to the cell surface expression of NKp46, CD56, and KLRG1 three subsets were observed: NKp46$^+$ ILC3-like cells were suggested to differentiate into ILC3 and some ILC1/NK-like cells, while KLRG1$^+$ILC3-like cells differentiated into ILC2[100]. However, the authors stressed that KLRG1$^+$ILC3-like cells could also generate other ILC subsets depending on the received stimulus[100]. Hence the phenotype of circulating ILC3-like cells might be independent of their potential to differentiate into various different cell type, but might depend on the received stimulus. Also, these differentiation experiments need to be

treated with caution, as most of the times cytokine secretion is the output of these experiments whereas the phenotype of the generated cells is not followed up. As ILCs show a high plastic dynamic, thorough studies are needed analysing not only secreted cytokines but phenotypic receptors (see Chapter 1.3).

1.3 Plasticity of ILCs

To make the world of ILCs even more difficult to understand, ILCs - independent of their origin - seem to be quite plastic[171], similar to CD4$^+$ T cells[172], as, at least *in vitro*, their phenotype and functionality changes depending on the surrounding cytokine milieu. It becomes more and more obvious that the functional capacity of ILCs might be strongly dependent on the kind of trigger that could can change the cytokine output completely. In this context, it is also important to distinguish between plasticity and differentiation. Within this thesis, plasticity is the ability of a mature cell to change their functional capacity, while differentiation indicates the change of phenotype when an immature cell differentiates into a mature cell. As immature cells have by definition no plastic capacity, we are focusing here on the plasticity of mature, tissue resident human ILCs. Data from murine studies will be mentioned explicitly (Figure 3).

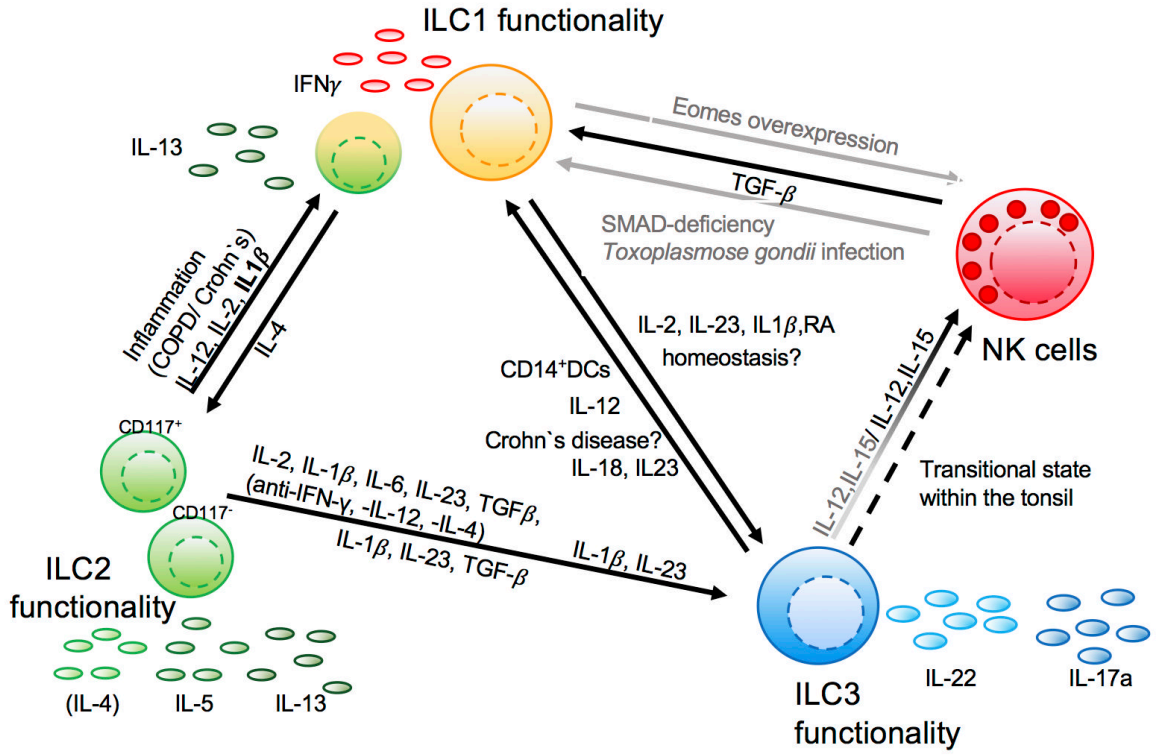

Figure 3: Summary of plasticity between the ILC subsets

It has to be mentioned that most studies focussed on changes in cytokine secretion but did not determine phenotypic changes. Hence, it is unknown if cells might have kept their phenotype. ILC1 data primarily refer to CD103$^+$ ieILC1, which largely resemble NK cells. Based on[99, 112, 173-185]. Legend: black arrows/ writing refers to human *in vitro* data, discontinued black arrows *ex vivo* data, grey arrow refers to murine data, grey and black arrow refers to human and murine data.

1.3.1 Plasticity of ILC1

1.3.1.1 ILC1-ILC3

Lamina propria derived human CD127^{+}ILC1 can be stimulated with IL-2, IL-23, and IL-1β to acquire an ILC3 phenotype, which is reversible, but potentially further enhanced by retinoic acid (RA) expressing CD14^{-}DCs[173]. Furthermore, an inversed correlation of ILC1 and ILC3 within tissue of squamous cell carcinoma patients has been observed[174] induced by IL-23-producing tumour cells[174].

1.3.1.2 ILC1-NK cells

Murine ILC1 showed a conversion into NK cells by the overexpression of the TF Eomes[175]. On the other hand a conversion of murine NK cells into an ILC1 phenotype was observed within SMAD4-deficient NK cells[186] and during *Toxoplasmose gondii* infection[177]. However, in humans this plasticity was not reported so far and mechanisms responsible for either conversion is not fully known[187]. Recently, a study showed the acquisition of a CD103^{+} ILC1 phenotype when human NK cells where stimulated *in vitro* with TGFβ and IL-15[184]. However, as discussed before it is questionable if this is really a plastic change or just the acquisition of a different phenotype from one NK subtype to another.

1.3.2 Plasticity of ILC2

1.3.2.1 ILC2-ILC3

Human ILC2s were first described to secrete IL-5 and IL13 mirroring a T_H2 cytokine profile[35] (Figure 2). However, human ILC2s can be divided into a CD117$^{-/lo}$ and a CD117^{+} population[112, 178], which have been recently described to differ in their functionality. CD117$^{-/lo}$ ILC2 were described to be more stable in their phenotype, as CD117^{+}ILC2 share the expression of CD117 and the TF Rorγt with ILC3[188]. In line with this, human CD117$^{-/lo}$ILC2s are more potent to secrete the T_H2 cytokines IL-5 and IL-13, when stimulated with IL-2, IL-25, IL-33, TSLP compared to CD117^{+}ILC2s[112]. Nevertheless, both ILC2 populations are able to secrete ILC3-associated cytokines, however they require different stimuli. Human CD117^{+}ILC2 were able to secrete IL-17A when stimulated either with IL-1β and IL-23 or with a non-physiological stimulation of IL-2, IL-1β, IL-6, IL-23, TGF-1β, anti-IFN-γ, anti-IL-12, anti-IL-4 (both ILC3-promoting conditions)[112, 178]. On the other hand, CD117$^{-/lo}$ ILC2 were not able to secrete ILC3-associated cytokines when stimulated with these cytokine combinations[112]. CD117$^{-/lo}$ILC2 required IL-1β, IL-23, and TGFβ for IL-17A secretion[178]. No IL-17A secretion was detected without TGFβ[178]. This conversion of a T_H2 cytokine profile into a T_H17 cytokine profile was in line with the down-regulation of corresponding TFs for each profile: the down-regulation of GATA-3 - associated with a T_H2 cytokine profile - paired with an up-regulation of Rorγt[178]-associated with a T_H17 cytokine profile. Of note, when comparing skin biopsies of healthy individuals to psoriatic patients, an increase in NK$_P$44^{-}ILC3 frequency was observed with a decrease in ILC2 frequency, which might indicate the potential of skin ILC2 to undergo a plastic change to an ILC3 phenotype during pathogeneses[178].

1.3.2.2 ILC2-ILC1

The antagonism of T_H1 and T_H2 cells is long known[189], within murine ILCs the inhibition of IFNγ production by binding of the TF Gata3 -the master TF in ILC2- to the *ifng* locus has been

shown[92]. Nevertheless, human ILC2 were observed to secrete IFNγ when stimulated with IL-12[179], which has been also observed within T cells[180]. In general, a change of a T_H2 cytokine profile into a T_H1 cytokine profile has been observed within patients with severe chronic obstructive pulmonary disease (COPD) and Crohn`s disease, which displays IL-12 signatures[179, 181, 182]. Upon deeper analyses, human CD117$^+$ILC2 is the subset able to secrete IFNγ when stimulated with IL-2, IL-1β, IL-12[112, 181]. Interestingly, IL-1β is necessary for the functional change of ILC2 into ILC1, as it has been proposed to prime ILC2 to environmental signals as well as enable plasticity[179, 190, 191]. However, this plasticity is not complete, as ILC2s lose their ability to produce IL-5, but are still able to secrete IL-13 in parallel to IFNγ[173, 179, 181]. A stimulation of the ILC2-to-ILC1 converted state with IL-4 erased the plasticity suggesting the specificity of this stimulation and an induced plastic effect[178, 181].

1.3.3 Plasticity of ILC3

1.3.3.1 ILC3-NK cells

One study observed the conversion of human tonsillar ILC3 and splenic murine ILC3, taken from humanized mice, into 'early differentiated NK cells` when cultivating ILC3 *in vitro* in the presence of IL-12 and IL-15, but not in the presence of IL-2 and IL-7[185]. ILC3 up-regulated the TF Eomes, showed common NK cell surface receptors and showed signs of cytotoxicity[185].

1.3.3.2 ILC3-ILC2

So far, no study highlighted a conversion of human ILC3 into ILC2. However, i*n vitro* IL-17-producing ILC3 from psoriatic lesions were able to convert back into CRTH2$^+$ILC2s in the presence IL-1β and IL-4[178]. One has to note that this conversion might rather be a reversal into original state rather than a differentiation, as ILC2 have been suggested to convert into ILC3 during psoriasis[178].

1.3.3.3 ILC3-ILC1

Several studies have described the secretion of IFNγ by human ILC3 suggesting the ability of ILC3 to convert into Group 1 ILCs. This is an interesting observation, as human ILC3 and NK cells share a common progenitor shown in *in vitro* differentiation studies[137]. ILC3s derived from several human tissues were able to secrete IFNγ after stimulation with IL-18 and IL-23[99]. Within the gastrointestinal system, CD14$^+$ DCs were reported to promote the conversion of ILC3 into CD127$^+$ILC1 by potential secretion of IL-12[173]. Additionally, the stimulation of ILC3 with IL-12 was also reported to result in a functional switch[183]. An increased frequency of ILC1s in contrast to reduced frequencies of ILC3 were observed in inflammatory bowel disease, especially Crohn`s disease[34, 192, 193]. It was suggested that ILC3 are the main ILC subset within homeostatic conditions, but that ILC1 frequencies increase at the expense of ILC3 due to a conversion during bowel inflammation[187, 194], however it remains questionable if this is a matter of plasticity or changes in ILC frequencies during Crohn's disease. Of note, upon treatment with anti-TNF or vedolizumab (anti-$\alpha_4\beta_7$-integrin) a slight decrease in ILC1 and increase in ILC3 frequencies was observed in Patients suffering from Crohn`s disease and ulcerative colitis[195].

Using t-SNE analyses of four different tonsillar populations, an *ex vivo* ILC3-ILC1 intermediate transition state was identified[29]. However, it has to be noted that the phenotype of described

ILC1 cells resemble more the phenotype of a $CD103^+$ieILC1 cell[29] - having a NK cell phenotype. Hence when looking at this data, one has to be careful, as hardly any researcher checked for the phenotype after stimulation. It might be that the cells have kept their phenotype[28, 99] or not. Furthermore, the missing ability to definitely distinguish human ILC1 and NK cells is further complicating the question of plasticity.

1.4 The development of NK cells and ILCs

The relationship of NK cells and ILCs is still a matter of intensive study and debate, especially within humans. Originally, ILCs and NK cells were thought to originate from the same precursor[27], whereas 10 years later their lineage relationship was viewed to be further apart[26]. However, a novel common ILC precursor (CILCP) generating ILC and NK cells has been recently identified in Id2-reporter mice[196]. Within reviews, murine and human data are often mixed together, which can create a biased picture, especially as murine and human ILCs are phenotypically different[197]. Here, I would like to highlight what is known from human (in comparison to murine) studies with respect to a common ILC and NK cell developmental pathway.

In human NK and ILC development a common lymphoid progenitor (CLP) defined as $CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^+$ and a common innate lymphoid precursor (CILCP) defined as $CD34^+CD45RA^+CD117^+IL1R^+Ror\gamma t^+$ have been described within SLOs, CB, and BM to give rise to all ILC and NK cell subsets[198, 199]. However, since CLP and CILCP were defined by different groups, it is currently not known if these cells are identical or if they mark two distinct precursor cell types. If the latter is true, potentially the CLP might give rise to CILCP by up-regulation of CD117. Immature $CD117^+CD127^+$ cells -phenotypical resembling tissue ILC3- within human PB have been identified and described to be able to differentiate into all ILC subsets as well as NK cells[81]. Hence, a model was proposed where circulating ILCs are able to migrate into tissues to mature into effector ILCs, called "ILC-poiesis"[170]. Additionally, a specific natural killer precursor (NKP) downstream to the CLP has been described as $CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$[199] (Figure 4).

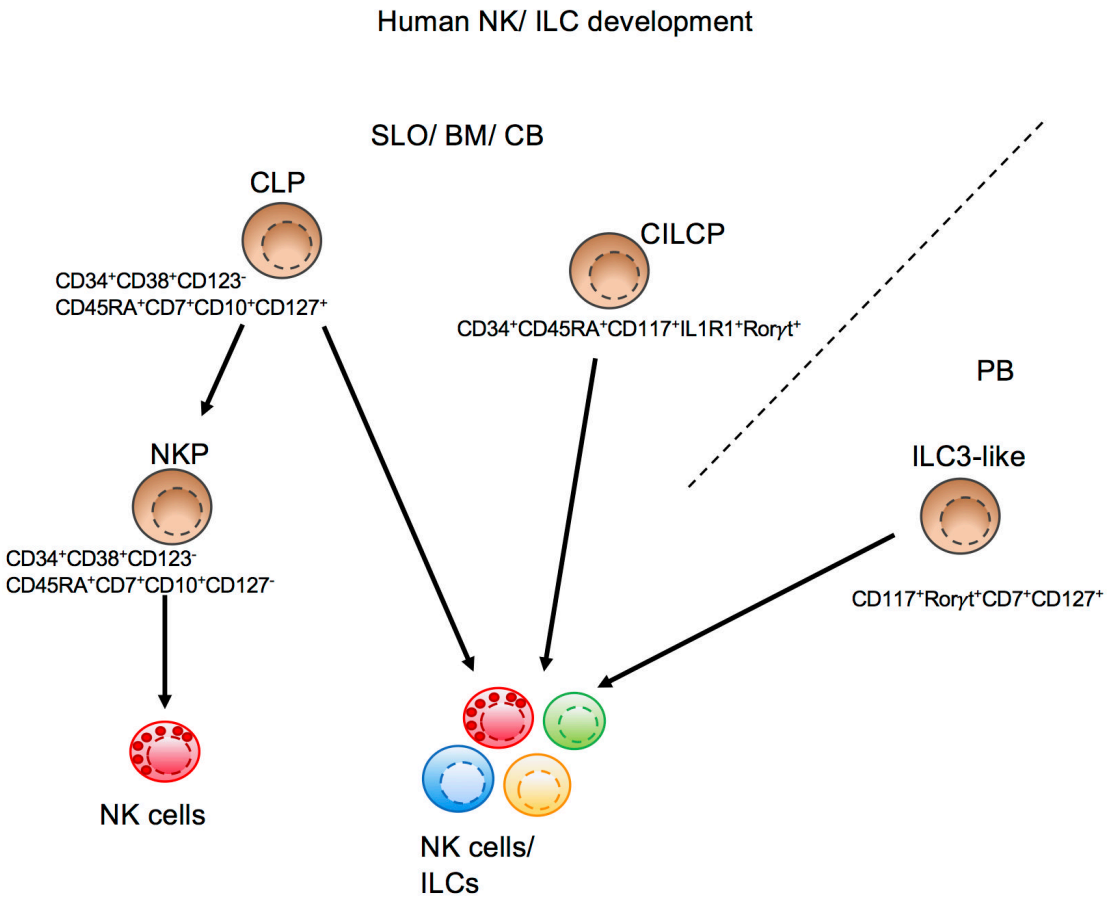

Figure 4: Human ILC and NK cell development

The scheme summarises current knowledge regarding the phenotype of human precursors of NK cells and ILCs. Abbreviations: common lymphoid progenitor (CLP), natural killer precursor (NKP), NK (Natural killer), CILCP (common innate lymphoid cell precursor), PB (peripheral blood), CB (cord blood), BM (bone marrow), SLO (secondary lymphoid organs). ILC (innate lymphoid cells), CD (cluster of differentiation). Graphic is based on the following references[81, 198, 199].

With the help of knock-out mice, murine reporter models, and cre-flox systems a similar hierarchy of ILC/NK cell precursors have been identified focussing more on TF expression than phenotypic features. A common CLP has been identified being able to give rise to T cells as well as ILC/NK cells depending on the TF expression[200]. To drive CLP into the ILC/NK cell direction, the TFs nuclear factor IL-3 induced (NFIL3 or E4BP4), thymocyte selection-associated high mobility group box protein (TOX), and T cell factor 1 (TCF1) are needed[200]. Down-stream of CLP, able to differentiate in all ILC subsets and NK cells, but not into T cells, the T cell factor (TCF) 1-dependent early ILC precursors (EILP) were identified[201]. Downstream of EILP still able to differentiate into NK and ILCs is the common ILC precursor (CILP) expressing $ID2^{+}ZBTB16^{+}$[196]. From this point on, ILC and NK cells need different transcriptional programs for differentiation. For NK cell differentiation, the TF TOX, ETS1, and Runt related transcription factor 3 (RUNX3) are needed resulting in a natural killer precursor (NKP)[26].

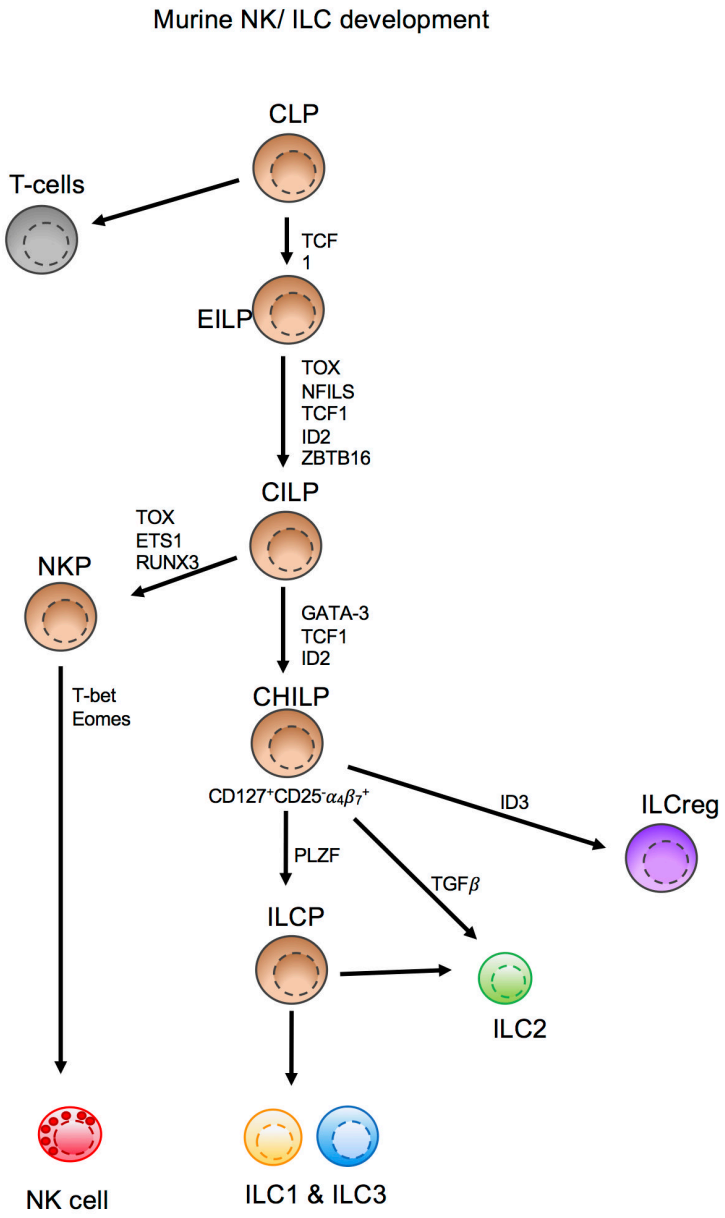

Figure 5: Murine NK and ILC development

The up to date identified murine precursors of NK cells and ILCs. Abbreviations: common lymphoid progenitor (CLP), early ILC precursor (EILP), natural killer precursor (NKP), NK (Natural killer), CILP (common innate lymphoid precursor), CHILP (common helper innate lymphoid precursor), ILCP (innate lymphoid cell precursor), ILC (innate lymphoid cells), CD (cluster of differentiation), ILCreg (regulatory ILC). Inspired by Vivier et al. (2018)[26] and based on the following references[26, 104, 196, 200-204].

This NKP can then further differentiate into NK cells via Eomes and T-bet expression[104]. For further development into ILCs, the CILP starts the expression of GATA-3, TCF-1, and ID2 to result in a 'common progenitor of all helper-like ILC subsets' (CHILP), which is defines as $Lin^{-}ID2^{+}CD127^{+}CD25^{-}\alpha_4\beta_7^{+}$[202]. CHILP further up-regulate the expression of the TF PLZF to become ILCP defined as $PLZF^{high}ID2^{high}GATA\text{-}3^{high}TOX^{+}$ able to generate ILC1, ILC2,

ILC3[203] depending on the TF used[200]. Of note, TGFβ was also described to enable ILC2 differentiation from CHILP[205]. Upon ID3 up-regulation, CHILP can also differentiate into regulatory ILCs (ILCregs)[204] (Figure 5). However, the human counterpart of CHILP has not been found yet.

It is important to mention that murine studies have highlighted the expression of the TF DNA-binding 2 (ID2) to be important for NK and ILC development, as NK and ILCs were observed to share a common $ID2^+$ progenitor[26]. ID2, as well as ID3 and ID4, are members of the basic helix loop helix (bHLH) TF family. The expression patterns of ID3 and ID2 significantly contribute to the lineage fate decision during precursor development. ID2 has been shown to be essential for the differentiation and survival of NK cells[206]. Interestingly, ID2-deficient mice were able to generate NK cells by upregulation of ID3, but these cells were not able to switch from a naïve to an effector phenotype[207]. The up-regulation of ID3 is thought to inhibit Notch transcription[208, 209]. Notch signalling is essential for cell commitment to the T cell lineage within the thymus. Hence studies could show a block in T cell commitment upon high ID3 expression[210, 211], which even promoted the differentiation into NK cells[211]. However, in progenitor cells already expressing the pre-T cell receptor (TCR) complex, ID3 overexpression lead to block in $\alpha\beta$ T cell development, but not in $\gamma\delta$ T cell development[212]. Human ILCs showed very low expression levels of ID2 *ex vivo*[98], however ID2 expression was higher in PB-derived common ILC progenitor (CILCP) $CD117^+$ cells compared to $CD34^+$ cells[81]. Nevertheless, it remains questionable if ID2 is indeed the defining TF for human ILCs.

1.4.1 Developmental stages of human NK cell differentiation

NK cells are thought to originate from the bone marrow (BM) and circulate from the periphery into secondary lymphoid nodes (SLNs) for further maturation[43]. Human NK cell development was originally divided into five different stages when starting from $CD34^+$ hematopoietic stem cells. Stage 1 ($CD34^+CD117^-$), where stage 1 is most probably the described NKP[199], stage 2 ($CD34^+CD117^+$), stage 3 ($CD34^-CD117^+CD161^+$), stage 4 ($CD34^-CD117^{+/-}$ $CD94^+CD161^+CD16^-CD56^{bright}$, here called $CD56^{bright}$ NK cells), which is further distinguished with their respective NKp80 expression, in stage 4a lacking NKp80 expression and stage 4b expressing NKp80[213], and stage 5 ($CD34^-CD117^-CD161^+CD94^{+/-}CD16^+CD56^{dim}$)[214]. Stage 5 might be further divided into two different cell types: in stage 5a $CD56^{dim}$ NK cells are still expressing NKG2A but can have additional expression of KIR, while within stage 5b, NKG2A expression is down-regulated while KIR expression is maintained and diversified[51] (Figure 6). An additional stage 6, containing terminally differentiated memory-like NK cells expressing CD57, has also been proposed[215, 216].

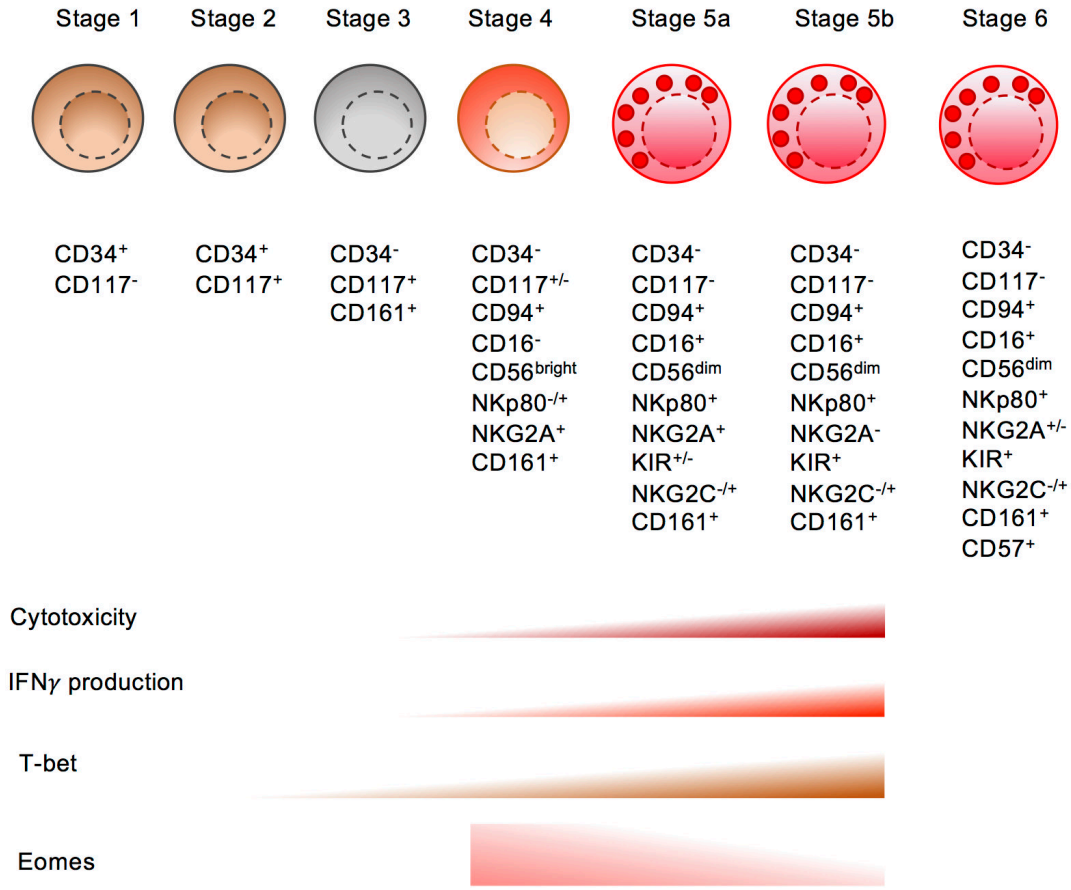

Figure 6: Overview on proposed human NK cell development

Overview of previously described stages of NK cell development: stages 1 to 6 with cell surface receptors, cytokines, and transcription factors[104, 213-215].

A unique feature of NK cells that sets them apart from all other ILCs is the ability to recognise HLA class I molecules in order to enable `missing self` recognition of aberrant and/or infected target cells. For this, CD56^{bright} NK cells primarily use the heterodimer CD94:NKG2A whereas further differentiation towards CD56^{dim} NK cells eventually leads to the generation of diverse KIR repertoires, which starts to diversify at stage 4 during NK cell development[215]. Upon target recognition, CD56^{bright} NK cells react with cytokine production whereas CD56^{dim} NK cells do not only produce cytokines but are also highly cytotoxic. *In vitro* and *in vivo* experiments have shown that the expression of NK specific receptors is a specifically timed process. The MHC 1B HLA-E-specific NKG2A molecule is upregulated first starting in stage 4 followed by the KIRs 2DL2/2DL3[217, 218] (depending on the KIR genotype) by some NK cells, followed by the acquisition of a diverse KIR repertoire[50]. The expression of HLA-C2-specific ligand 2DL1 *in vivo* and *in vitro* has been described to be delayed[73, 217]. Interestingly, analyses of NK cell repertoires in CB NK cells revealed an unbiased KIR expression pattern[219] suggesting that KIR expression is biased post-birth. CD56^{dim} NK cells can further mature, which involves the down-regulation of NKG2A with continued and diversified KIR expression leading to NK cells expressing several different KIR in a clonally-distributed way (NKG2A^{-}KIR^{+} NK cells)[51]. The

different NK cell stages are not only distinguished by phenotypic differences, but also exhibit different patterns of T-bet and Eomes expression. During the developmental stages 3 to 5, NK cells mature from an Eomes^{high} T-bet^{low} to an Eomes^{low} T-bet^{high} phenotype[220].

According to the NK cell developmental stages and evidential experiments, CD56^{bright} NK cells are thought to be the precursors of CD56^{dim} NK cells due to the following evidence: CD56^{bright} NK cells are less mature compared to CD56^{dim} NK cells[221], more abundant in neonatal blood compared to PB and lymph node (LN)-derived CD56^{bright} NK cells have longer telomerases compared to CD56^{dim} NK cells[222, 223], and *in vitro* IL-2-stimulated CD56^{bright} NK cells from SLNs became cytotoxic with up-regulation of CD16 and KIR expression[47]. The conversion of CD56^{bright} NK cells to CD56^{dim} NK cells might also require distinct environmental stimuli, as synovial fibroblasts were described to be needed for differentiation[223]. Furthermore, CD56^{bright} NK cells dominate the early period after HSCT[224]. Two studies, one using CyTOF analyses with pseudo time projection and the other using scRNA-seq showed transitional stages between CD56^{bright} NK cells and mature CD57^{+} CD56^{dim} NK cells[225, 226] further suggesting a linear relationship between CD56^{bright} and CD56^{dim} NK cells.
Nevertheless, there is also evidence against the hypothesis that CD56^{bright} NK cells are precursors to CD56^{dim} NK cells and might instead constitute an independent and stable subset with unique features. CD56^{bright} NK cells and CD56^{dim} NK cells have different, unique responsibilities within the immune system already suggesting two unique identities. PB of *GATA2*-deficient patients showed a reduced, but detectable CD56^{dim} NK cell population, while CD56^{bright} NK cells were completely absent[227] suggesting a stronger dependence of CD56^{bright} NK cells on GATA-2 compared to CD56^{dim} NK cells. Furthermore, if CD56^{bright} NK cells are the only CD56^{dim} precursor, CD56^{dim} NK cells were also absent within the *GATA2*-deficient patient, however within this patient CD56^{dim} NK cells were only reduced. Even though different *in vivo* stages were identified[214], no commonly used *in vitro* protocol cultivating stage 1- 3 precursors or CD56^{bright} NK cells (stage 4) on stromal cell lines led to a down-regulation of CD56 or NKG2A[213, 228] and hence a more mature NKG2A^{-}KIR^{+} NK phenotype, as seen *in vivo*. This findings question, if CD56^{bright} NK cells are indeed the direct precursor from CD56^{dim} NK cells, as this complex KIR repertoire cannot be achieved by culturing CD56^{bright} NK cells. However, as NK cell development takes place within SLNs[214], tissue CD56^{bright} NK cells might in fact be the main CD56^{dim} NK cell precursor, while peripheral CD56^{bright} NK cells, which are used in the large majority of studies, might have lost the ability to downregulate NKG2A, upregulate KIR, and rather remain as a unique subset in the periphery. Importantly, within this thesis it was possible to identify a unique CD56^{-}CD117^{-} NK cell precursor within umbilical cord blood (CB), which differentiates into NK cells exhibiting high KIR expression in the absence of NKG2A, a typical feature of stage 5b of NK cell development (see Chapter 2).
Of note, an alternative NK developmental pathway within the murine thymus has been proposed to generate a unique NK cell subset, but could so far not been identified within humans[229].

1.4.2 Developmental stages of human ILCs

While distinct developmental stages have been experimentally verified in murine and human NK cell studies, the developmental pathway of ILCs remains largely elusive. It has been suggested that the development into the different human ILC subsets might be purely based on

their individual TF expression. By following this thought, ILC1 require TBET to develop from ILCP[230], ILC2 require GATA-3, and ILC3 RORγT respectively. In line with this, PB CD117$^+$ ILCp were shown to give rise to all three ILC subsets as well as NK cells[81]. By further analyses of PB CD117$^+$ cells, three different CD117$^+$ subsets were identified by respective CD56, NKp46, and KLRG1 expression[100]. In this study, KLRG1$^+$CD117$^+$ cells dominantly differentiated into ILC2[100], while NKp46$^+$CD117$^+$ cells were able to differentiate in ILC3 or Group 1 ILCs depending on the given stimulus[100]. Another cell surface receptor distinguishing lineage development between ILC3, NK cells and ILC2 is CD56[188]. A murine model also identified transient GATA-3 expression to be important for the development of the individual ILC subsets[231]. A recent *in vitro* transfection study of human CD34$^+$ with *GATA3* messenger RNA confirms the significant increase in ILCp by forced GATA-3 expression[232], while the transient overexpression of RORC did not enhance ILCp or ILC3 development in this study but rather decreased it. This is interesting, as a human CD34$^+$Rorγt$^+$ precursor has been identified within tonsils and lamina propria giving rise to IL-22-secreting ILC3, but not NK cells, while CD34$^+$Rorγt$^-$ were able to differentiate into ILC3 and NK cells[233]. RORC-deficient patients had reduced PB ILC3 frequencies and no ILC3/ T_H17 effector function during Candida infection[141] indicating that RORC is important for ILC3 maturation or effector function. Interestingly, when intestinal biopsies obtained from IBD Patients are treated with RorγT inhibitors hardly any T_H17 cells were detectable, but normal ILC3 frequencies exhibiting no decrease in effector function[140]. Combining these two studies suggest a developmental dependency of ILC3 on RORC, but also showed that once within the tissue, ILC3 retain their effector function even if RORC is inhibited.

All in all, it seems as if ILC3 and NK cells are more closely related to one another than to ILC2, which is in line with *in vitro* studies for NK and ILC development discussed in the next Chapter (1.5.3). Interestingly, the development of ILC1 is an area not well studied within literature.

1.4.3 *In vitro* models for NK and ILC development

After identifying an IL-22-secreting CD56$^+$NKp44$^+$ subset within the human MALT[134, 135], previously called NK-22 cells, now called ILC3, a similar population was identified when co-cultivating human umbilical cord (CB) CD34$^+$ hematopoietic stem cells with murine stromal cells and with interleukins favouring the generation of NK cells[137]. These cells were able to secrete IL-22 when stimulated with IL1β/ IL-23, which was enhanced by adding TL1A the ligand for death receptor 3[234]. These IL-22-producing cells had a phenotypic resemblance to stage III NK cell progenitors (CD56$^+$CD117^{high}CD94$^-$)[138, 235]. However, due to the different cytokine profiles with ILC3/ NK-22 secreting IL-22, while NK cells secrete IFNγ, they were thought to be distinct from each other. Furthermore, the two different populations needed different cytokine requirements, as the absence of IL-15 lead to a block in NK cell development, while in the absence of IL-7 and stem cell factor (SCF) a block of ILC3 was detected[137]. Nevertheless, it was possible to develop ILC3 and NK cells within the same *in vitro* conditions, therefore a common precursor of NK cells and all ILC subsets has been proposed, which is today still in line with current literature (see Chapter 1.4). The generation of ILC3 was only shown on murine stromal cells so far, but the generation of human ILC3 on human stromal cell lines might bear interesting therapeutic potential as shown in this thesis (see Chapter 2.2).

It was further possible to generate human ILC2 from thymic CD34$^+$CD1a$^+$ precursor cells and tonsillar CD117$^+$CD56$^-$ cells when co-cultivation on stromal cells expressing NOTCH

ligands[188, 236], suggesting different developmental requirements for the individual ILC subsets. Interestingly, no *in vitro* condition is known to generate human ILC1 from common $CD34^+$ hematopoietic stem cells. This might be due to the fact that most studies analysed the cells merely functionally[198], with ILC1 and NK cells both being able to secrete IFNγ. So far, no clear-cut phenotype has been described for ILC1, especially in *in vitro* culture.

1.5 Cord blood – a valuable source

Amongst other sources, human $CD34^+$ hematopoietic stem cells needed for *in vitro* differentiation assays, as described in Chapter 1.4.3, can be purified from umbilical cord blood (CB). CB is a very interesting source, as it is ethically safe, has lower infectious risk compared to PB, and CB cells are easily accessible. Due to all these features, CB research became very popular in the 1980s. In 1982, Hal Broxmeyer felt that discarding CB after birth was a waste, as CB might be a useful source for progenitor cells useable for transplantation. He and others founded the Biocyte Corporation for the thorough study of CB for HSCT use[237]. Of note, before the 1980s, typical sources for HSCT were progenitor cells from BM or G-CSF mobilized PB from HLA- matched donors, most likely siblings. Over the years, CB proved to be a useful source of progenitor cells due to high availability, quality, and stability of these precursors even three days post-birth [238, 239]. CB was found to harbour high amounts of $CD34^+$ hematopoietic stem cells, which were known to give rise to various different immune cell lineages[240]. This feature was very useful to the scientific community, as it made the *in vitro* generations of T, B, and NK cells possible[137, 235, 241, 242]. Furthermore, CB cells showed high proliferative capacity, even in single cell cultures[239], which was seen as indicator for the ability of CB cells to repopulate the recipient immune system after transplantation[243]. As technologies improved, $CD34^+$ hematopoietic stem cells were further characterised and it became obvious that successful engraftment of recipients not only depends on $CD34^+$ frequency, but also on the composition of the different $CD34^+$ subsets with the developmental potential and associated phenotypic differences[244-246]. Besides precursors, CB already contains various different immune cell populations similarly found in PB post-birth. Even though CB immune cells phenotypically resemble adult immune cells, CB T cells, NK cells, and B cells were overall more naïve and exhibited lesser functional capacity compared to PB[239, 243, 245, 247-249]. A very recent study highlighted that the immunity present within CB shows differences to post-birth immunity and that the immunity of term infants (being born $\geq$ 37 week of gestation) is already different from that to preterm infants (being born < 30 week of gestation). Furthermore, this study highlights the adaptation of our early immune system towards the environmental influences very early in life[4]. Due to the various different precursor populations, CB is highly attractive to get a deeper understanding of the immune system's development, to identify novel precursors, and for potential therapeutic use, such as for HSCT.

The first transplantation using CB as graft source was conducted in 1988 with a child suffering from Fanconi`s anaemia, who received CB from his unaffected sibling[250]. Several studies focussed on CB as graft source especially within children, which proved to be safe between siblings[238, 250, 251], but also between unrelated donors[252, 253].

It is important to stress that HSCT in children and adults shows differences in terms of patient conditioning, hematopoietic recovery and immune responses, and thus needs to be analysed separately. Matched or mis-matched HSCT transplants have been shown to reconstitute the

immune system of children without significant differences in adverse effects, whereas adults receiving a mismatch transplant showed slower immune reconstitution with increased risks of opportunistic effects[254]. Hence, not all studies are applicable to one or the other setting.
After transplantation the immune system needs time to recover until it reaches the initial immune cell counts comparable to healthy individuals. This leads to an impaired, not fully competent immune system in the recipient within the first few months after HSCT[255]. This reconstitution of the immune system is a critical time point, as the risk of adverse effects is high. Patients can suffer relapses due to various different reasons, can get infections due to an impaired immune system or are attacked by the donors alloreactive T cells leading to GvHD, which is responsible for high morbidity and mortality[72]. Hence, the monitoring of the reconstitution of the patients' immune system is of vital importance. The individual immune cell populations have been observed to follow distinct time lines of reconstitution. NK cells are the first lymphocyte population rapidly recovering after several weeks after HSCT[256] and especially the rapid recovery of $CD19^+$B cells and NK cells after CB transplantation resulted in reduced mortality risk[74]. NK cells in particular play an important role, as mismatched donor NK cells were beneficial for a GvL effect[257] potentially reducing a risk of relapse. A recent study compared the reconstitution capacity of PB, CB, and BM as graft sources in over 300 adult patients suffering from haematological malignancies[258]. Patients reconstituted with CB showed significant higher frequencies of $CD3^-CD56^+$ and $CD16^+CD57^-$ NK cells as well as different $CD4^+$ T cell subsets at day 100 after HSCT as well as very high frequencies already within the early phase after reconstitution. $CD8^+$ T cells on the other hand were significantly lower and their reconstitution was delayed compared to BM and PB transplanted patients[258]. A lower incidence rate of chronic GvHD was observed in patients with higher frequencies of regulatory T cells and $CD16^+CD57^-$ NK cells, while a higher incident rate correlated with elevated $CD8^+$ T cell frequencies[258]. Hence, CB as graft source seems to reduce the risk of GvHD in adult patients, which was already seen in paediatric patients[239, 253, 259]. Of note, especially interesting was the study in respect to the overall reconstitution of NK cells after PB, CB, or BM engraftment, as the most immature source -CB- showed the fasted engraftment of NK cells with very high frequencies of mature ($CD16^+CD57^-$) NK cells already after 100 days after reconstitution, which was maintained even after 2 years[258]. These findings indicate a unique role for CB as graft source for the reconstitution of mature NK cells.

1.6 What is known about ILCs in cord blood?

CB is a highly versatile source for ILC research, as all three ILC subsets (ILC1, ILC2, and ILC3) as well as $CD56^{bright}$ and $CD56^{dim}$ NK cells can be identified with significant higher ILC frequencies compared to PB[42]. Additionally, studying CB precursors and their potential can also generate more insight into the developmental pathway and relationship of human ILCs. Despite these advantages, so far, no publication from other groups entirely focused on CB ILCs. CB ILC data was mainly used for method validation[99] and in most cases buried in the supplementary data section[81, 99]. In general, CB ILCs seem to be more similar to PB ILCs than compared to tissue ILCs. Like PB ILC3, CB ILC3 were shown to differentiate in all ILC subsets and NK cells[81]. PB and CB ILC2 exhibited a comparable functionality[99], while PB and CB ILC3 expressed low levels of ectoenzymes compared to tissue ILC3s[157]. The cell surface receptor CD5 was found to be expressed in functionally immature CB ILCs compared to mature CB ILCs[98]. Furthermore, evidence exists that gender influences ILC frequency, as ILC2

frequency is higher in male than female neonates[260]. Importantly, no transcriptome analysis of CB ILCs is available so far, therefore it is not known if CB ILCs are similar to PB ILCs or represent a unique cell population. All in all, despite the high ILC frequency within CB, direct transcriptome analyses comparing all three ILC subsets and a thorough analyses of the functional and phenotypical properties of CB ILCs are still lacking.

1.7 Aim of this thesis

The aim of this thesis was to determine developmental stage, function, and phenotypic characteristics of ILCs within human neonatal blood. CB reflects the state of the neonatal immune system and thus provides a high valuable source for stem cells and naïve effector cells in various clinical settings including haematological malignancies. So far, detailed studies on neonatal human ILCs have been largely lacking. Within this study, we first generated the necessary tools to faithfully identify ILCs within CB by designing a flow cytometry panel to identify CB ILCs with a lineage cocktail especially adapted to exclude all unwanted cell populations (see Chapter 2.1). This is the first crucial step as the lineage cocktail is especially important to gate out unwanted cell populations and can lead to impurities within the individual ILC subsets, especially within CB ILC1 lacking most cell surface receptors. The next step was to analyse if functionality and phenotype of CB ILCs is comparable to described tissue and/ or already described peripheral ILCs (see Chapter 2.3 & 2.4). A further important aim was to analyse the developmental stage of CB ILCs. To this end, a novel method for *in vitro* generation of NK cells from $CD34^+$ cell on MSCs that was recently developed in our laboratory, was adapted for analyses of ILC differentiation. So far, the generation of NK cells and ILCs was only described on murine stromal feeder cells, but not on human cells, and we wanted to test if we are able to generate *in vitro* ILC3 and NK cells with the help of human feeder cells. As especially human ILC3 have been described to be beneficial in various types of diseases, such as inflammatory bowel disease, the generation and deep analyses of the *in vitro* generated ILC3 might bear high therapeutic potential (see Chapter 2.2). Finally, this thesis attempted to provide an overview of the translational relevance and the future potential of human ILCs to be used within clinical settings (see Chapter 2.5).

2 Publications

2.1 Characterisation of Human Innate Lymphoid Cells from Neonatal and Peripheral blood

Bennstein SB[*], Manser AR[*], Weinhold S, Scherenschlich N and Uhrberg M (2019). OMIP-055: *Characterization of Human Innate Lymphoid Cells from Neonatal and Peripheral blood*. Cytometry Part A. Doi: 10.1002/cyto.a.23741, [*] contributed equally

Impact factor Cytometry Part A: 3.124

Contribution to this publication:

- Performing experiments
- Establishment of the lineage cocktail
- Critical discussion of antibodies and fluorophores used for faithful detection of ILCs
- Writing the manuscript
- Revision of the manuscript

Studying human ILCs is challenging, as ILCs are very rare. Moreover, not all tissues contain all five subsets of described ILC subtypes: ILC1, ILC2, ILC3, $CD56^{bright}$ NK cells, and $CD56^{dim}$ NK cells[82] making it difficult to directly compare all subtypes. Within this study we have developed a staining panel for faithful identification of human ILCs and NK cells within CB. CB is a valuable source when studying ILCs, as ILC frequencies are significant higher compared to PB[42] and CB contains many precursors potentially harbouring not yet identified ILC precursors. We developed a novel protocol to accommodate the unique composition of progenitor and mature cell types in CB and adapted the lineage cocktail to include CD235a for erythrocyte precursors, which resulted in a faithful 8-colour staining panel. This study also included recommendations for ILC staining within PB and tonsils.

2.2 HCMV infection in a mesenchymal stem cell niche: differential impact on the development of NK cells versus ILC3

Ising R, Weinhold S, Bennstein SB, Zimmermann A, Degistirici Ö, Kögler G, Meisel R, Hengel H, Timm J and Uhrberg M (2020), 9(1): *HCMV infection in a mesenchymal stem cell niche: differential impact on the development of NK cells versus ILC3***.** Journal of Clinical Medicine. Doi: 10.3390/jcm9010010

Impact factor Journal of Clinical Medicine: 3.303

Contribution to this publication:

- Performing the analyses of ILC3 within HCMV-uninfected cultures
- Figure preparation
- Writing the manuscript
- Revision of the manuscript

HCMV infection is a global burden and changes NK cell repertoires by switching the expression of the heterodimer CD94:NKG2A into CD94:NKG2C, leading to the expansion of $NKG2C^+$ NK cell in most HCMV-infected individuals. However, so far, no *in vitro* system was available to study the effect on NK cell development during an acute HCMV infection. We have previously established an *in vitro* system using human mesenchymal stem cells (MSCs) to generate NK cells from $CD34^+$ hematopoietic precursors[228]. Within this study, we compared the NK cell generation on HCMV-infected MSCs to that on uninfected MSCs. We could show that NK cell frequencies are not affected by an acute HCMV-infection, however the NK cells generated on HCMV-infected MSCs showed a slightly more mature phenotype and significant higher effector function in respect to cytokine production. Furthermore, a significant increase in $CD16^+NKG2C^+$ NK cells were detected, but no NKG2C expansion as seen *in vivo* was observed.
Besides NK cells, it is published that ILC3s are generated within these *in vitro* cultures as well. Within this study, we observed the generation of $CD94^-CD56^+$ cells on HCMV-uninfected MSC, which were significantly decreased within HCMV-infected cultures. A loss of ILCs, especially ILC3, have already been seen within (Human immunodeficiency virus) HIV infections[105, 261], but not within HCMV infections. We further validated that the generated $CD94^-CD56^+$ cells corresponded to an ILC3 phenotype by intracellular staining of their described master transcription factor Rorγt as well as staining for the cell surface molecules NKp44 and RANKL. When subjected to cytokines known to induce IL-22 production in *bona fide* tissue ILC, we observed most *in vitro* generated ILC3 secreted IL-22. Hence, this study is the first to generate functional human ILC3s mirroring tissue ILC3 within a fully human *in vitro* system usable for Good Manufacturing Practice (GMP) conditions. This is especially of interest, as circulating ILC3s differ in their characteristics from tissue ILC3 and do not produce IL-22[81] (see Chapter 2.4). Moreover, the presence of IL-22-secreting tissue ILC3 has been associated with a reduced risk of GVHD[164] by protecting intestinal stem cells via IL-22[159]. Hence, this study constitutes a first step towards generating human IL-22-producing ILC3 for therapeutic purposes.

2.3 Umbilical cord blood-derived ILC1-like cells constitute a novel precursor for mature KIR$^+$NKG2A$^-$ NK cells

Bennstein SB, Weinhold S, Manser AR, Scherenschlich N, Noll A, Raba K, Kögler G, Walter L and Uhrberg M (2020), *Umbilical cord blood-derived ILC1-like cells constitute a novel precursor for mature KIR$^+$NKG2A$^-$ NK cells.* eLife 2020;9:e55232 DOI: 10.7554/eLife.55232

Impact factor eLife: 7.080

Contributions to this publication:

- Performing most experiments
- Conception of the experimental design and initial research question
- Data analyses
- Figure preparation
- Writing of the manuscript
- Revision of the manuscript

In the beginning of this study, a clear-cut distinction between ILC1 and NK cells was generally accepted based in murine data showing differential TF expression pattern including Eomes and Tbet. However, discrimination between human ILC1 and NK cells became more and more challenging. Classical NK cell surface molecules e.g. CD94 were no longer regarded as exclusive attributes to NK cells alone, but also became part of the ILC1 description, thus blurring the borders between both cell types. In our study, we provided the first transcriptomic comparison of neonatal ILC1-like cells to NK cells revealing a clear separation of ILC1-like cells from NK cells by global transcriptional analyses, such as PCA, and could also show high expression of T cell-associated molecules, such as *CD5, CD6*, *CD28* on ILC1. We observed four different ILC1-like populations based on CD5 and CD161 expression harbouring different additional T cell associated molecules on the cell surface. Upon stimulation with IL-12/ IL-18, we observed IFNγ production only within the small CD161$^+$ fraction, while the large majority of CD161$^-$ ILC1 remained unresponsive indicating an immature phenotype. Due to these observations, we refer to neonatal ILC1 as ILC1-like cells, as they phenotypically, but not functionally resemble ILC1. Next, we considered the possibility that ILC1-like cells might be in fact NK or T cell precursors. We therefore sorted ILC1-like cells and incubated them for 14d on the murine stromal feeder cell line OP9-DL1 in either T cell or NK cell differentiation conditions. We could not detect any differentiation or survival of ILC1-like cells within the T cell condition, whereas we observed a faithful differentiation of neonatal and with less efficiency PB ILC1-like cells into NK cells including up-regulation of NKG2A and KIR.

Notably, these *in vitro* generated ILC1-like derived NK cells exhibited a complex KIR repertoire and to our knowledge represent the first *in vitro* generated NK cells that differentiated to the advanced mature state of NKG2A$^-$KIR$^+$ NK cells. We could show that ILC1-like derived NK cells were cytotoxic based on granzyme and perforin expression, the ability to recognize HLA-deficient target cells ('missing self'), and recognition of antibody coated target cells (ADCC). Based on our data, we concluded ILC1-like cells to be a novel NK cell precursor (NKP) potentially migrating from neonatal blood to the tonsils to become NK cells. In line with this assumption, we observed a significant frequency decline of ILC1-like cells with increasing

gestational age. In contrast, ILC2 and ILC3 frequencies remained unchanged during gestation, but exhibited a significant decrease with increasing age. Moreover, ILC1, showed a distinct chemokine receptor pattern including CCR7, CCR4, and CCR9 enabling tissue migration. This study suggests that both ILC1-like cells and $CD56^{bright}$ NK cells contribute to the development of $CD56^{dim}$ NK cells. While ILC1-like cells might majorly contribute to the generation of KIR repertoires by differentiation into a $KIR^{+}NKG2A^{+/-}$ $CD56^{dim}$ population, $CD56^{bright}$ NK cells might contribute to the less mature $KIR^{-}NKG2A^{+}$ $CD56^{dim}$ population. In summary, the study opens novel insights into human NK cell development and contributes to an emerging picture indicating that circulating ILCs are a rich source for precursors of NK cells and ILCs.

2.4 Transcriptional and functional characterization of neonatal circulating ILCs

Bennstein SB, Scherenschlich N, Weinhold S, Manser AR, Noll A, Raba K, Kögler G, Walter L and Uhrberg M (2021). *Transcriptional and functional characterization of neonatal circulating ILCs.* STEM CELLS Translational Medicine, in press

Impact factor Stem Cell Translational Medicine: 6.429

Contributions to this publication:

- Data analyses via R
- Functional and phenotypic analyses of ILCs
- Conception of the experimental design and research question
- Figure design
- Writing the manuscript
- Revision of the manuscript

Within this study, we generated the first transcriptomic data set of human umbilical cord blood (CB) ILCs. We observed a clear-cut and distinct phenotype within CB ILCs compared to NK or T cells by principle component analyses (PCA). Interestingly, ILC1 clustered closer to T cells than NK cells, while ILC3 clustered closer to NK cells. When we compared the transcriptomic signature of each ILC subset to $CD56^{bright}$ NK cells, we observed a set of shared up-regulated genes within all three ILC subsets as well as unique genes for each subset. Within the shared genes we detected the chemokine receptor *CCR4*, *CD28*, *SLAMF1*, and the transcription factor (TF) *ID3*, resulting in a unique *ID3/ID2* ratio > 1 in all CB ILCs, but not in CB NK cells or tonsillar ILC3. This was an unusual finding, as murine and human ILCs were previously described to be dependent on ID2 and not ID3. The only other subset having this unique ratio were CB $CD4^+$ T cells, which might suggest that they share a developmental pathway with CB ILCs. We further analysed the only previously published PB ILC transcriptomic data set of peripheral blood ILC1, ILC2, as well as ILC3 and observed that this unique *ID3/ID2* ratio >1 was not seen within PB ILCs and neither in PB $CD4^+$ T cells, suggesting a unique transcriptional regulation of ILCs pre-birth. We further characterized the transcriptome of each CB ILC subset in comparison to each other and observed novel genes for each subset. In this context, another novel TF, *ZTBT46,* caught our attention, which was one of the most highly expressed genes within ILC3-like cells. Upon specific stimulation with previously described cytokines, we observed only CB ILC2 and NK cells to exhibit functionality similar to their PB and tissue counterparts, while ILC1-like and ILC3-like cells showed hardly any functionality (except for LIF production within ILC3-like cells). As we have previously shown that ILC1-like cells were able to differentiate into NK cells (see Chapter 2.3) we focused in this study on ILC3-like cells. We were wondering if CB ILC3-like cells might be able to react to alternative stimuli beyond the established cytokine-mediated stimulation. In this regard, we observed high read counts by RNA sequencing for the TLR chains 1 and 2. When stimulating sorted ILC3-like cells with IL-2 with or without the TLR2:1 ligand Pam_3CSK_4, we indeed detected significant higher proliferation and secretion of various cytokines in the presence of Pam_3CSK_4 suggesting a unique role of ILC3-like cells for host defence pre-birth. All in all, we were able to show that CB ILCs have a unique transcriptional

identity compared to PB and tissue ILCs and that CB ILCs might share a developmental pathway with $CD4^+$ T cells potentially originating from the thymus.

2.5 Biology and therapeutic potential of human innate lymphoid cells (ILCs)

Bennstein SB and Uhrberg M. *Biology and therapeutic potential of human innate lymphoid cells (ILCs). The FEBS Journal*, Invited State-of-the-Art-Review, submitted

This study is an invited Review that was submitted to The FEBS Journal belonging to John Wiley & Sons. As author the re-use of the article in a new publication does not need prior permission. Wiley Online Library generally allows the usage of this paper within this thesis if cited properly.

Impact factor *The FEBS Journal*: 4.739

Contributions to this publication:

- Literature search
- Discussing the Review topic
- Conception of the Review
- Figure design
- Writing the manuscript

ILCs have been identified within the last decade to be important players in mucosal barrier functions, tissue homeostasis, and inflammation. Until today more studies were conducted using murine model systems, but recent advances were also made for human ILCs. In this State-of-the-Art-Review, we summarize how this novel knowledge on human ILCs contributes to different disease burdens, in particular virus infections, tumours, and autoimmunity. Interestingly, the three ILC subsets play ambiguous roles within the three disease burdens. We also highlight how human ILCs might be used for novel therapeutic approaches.

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4 Thesis relevant publications

4.1 Conference talks

1st Virtual meeting of the Young Immunologist, DGFI, 2020
Deep characterization and functional analyses of neonatal and peripheral innate lymphoid cells (ILC) subsets

Novel Concepts of Innate Immunity 2019 in Tübingen, Germany:
Characterization of innate lymphocytes in cord blood reveals a novel ILC1 population with NK cell differentiation potential

7th Retreat of the Düsseldorf School of Oncology (DSO), Düsseldorf:
Characterization of innate lymphocytes in cord blood reveals a novel ILC1 population with NK cell differentiation potential

6th Retreat of the Düsseldorf School of Oncology (DSO), Düsseldorf:
Role of innate lymphoid cells (ILC) in treatment for acute leukaemia in children

4.2 Conference posters

53. Jahrestagung der Deutschen Gesellschaft für Transfusionsmedizin und Immunhämatologie, 2020, digital
Deep characterization and functional analyses of neonatal and peripheral innate lymphoid cell (ILC) subsets (ePoster).

18th Meeting of the Society for Natural Immunity 2019, Luxembourg
Neonatal blood contains an ID3^{+}CD117^{-} NK cell progenitor that preferentially differentiates into KIR^{+} NK cells

Cord blood connect: The international Congress for Cord Blood and Perinatal Tissue Research 2019, Miami, USA (presented by Prof. Dr. Gesine Kögler)
Characterization of Innate Lymphocytes in Cord Blood Reveals a Novel ILC1 Population with Natural Killer Cell Differentiation Potential

4.3 Publications

1. **Bennstein SB,** Scherenschlich N, Weinhold S, Manser AR, Noll A, Raba K, Kögler G, Walter L and Uhrberg M. Transcriptional and functional characterisation of neonatal circulating ILCs. STEM CELLS Translational Medicine- in press, 2021

2. **Bennstein SB**, Weinhold S, Manser AR, Scherenschlich N, Noll A, Raba K, Kögler G, Walter L and Uhrberg, M. Umbilical cord blood-derived ILC1-like cells constitute a novel precursor for mature KIR^{+}NKG2A^{-} NK cells. *eLife,* 9**:** e55232, 2020.

3. Ising R, Weinhold S, **Bennstein SB**, Zimmermann A, Degistirici Ö, Kögler G, Meisel R, Hengel H, Timm J and Uhrberg M (2020), 9(1). *HCMV infection in a mesenchymal*

*stem cell niche: differential impact on the development of NK cells versus ILC3***.** Journal of Clinical Medicine.

4. **Bennstein SB,** Manser AR, Weinhold S, Scherenschlich N, Uhrberg M. OMIP-055: Characterization of Human Innate Lymphoid Cells from Neonatal and Peripheral Blood. Cytometry Part A. 2019;0(0).

5 List of abbreviations

Abbreviation	Explanation
ADCC	Antibody dependent cell-mediated cytotoxicity
AHR	Aryl hydrocarbon receptor
ALL	Acute lymphoblastic leukaemia
APC	Antigen presenting cell
CB	Cord blood
CCL	CC chemokine ligands
CCR	CC chemokine receptor
CD	Cluster of differentiation
CILCP	Common innate lymphoid cell precursor
CLP	Common lymphoid progenitor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DC	Dendritic cell
DN	Double negative
DNA	Deoxyribonucleic acid
Ds	Double stranded
EILP	Early ILC precursors
Eomes	Eomesodermin
FASL	FAS ligand
FDC	Follicular dendritic cells
GvHD	Graft-versus-host disease
GVL	Graft-versus-leukaemia
HEV	High endothelial venules
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
ICAM-1	Intracellular adhesion molecule 1
ID2	DNA binding 2
ID3	DNA binding 3
IFNγ	Interferon gamma
IL	Interleukin
ILC	Innate lymphoid cell
iNKT	Invariant natural killer T cells
KLRB1	Killer cell lectin-like receptor subfamily B
LFA-1	Leukocyte function-associated antigen 1
Lin	Lineage
LN	Lymph node
LT	Lymphotoxin

LTi	Lymphoid tissue inducer cells
MadCAM1	mucosal vascular addressing cell adhesion molecule 1
MALT	Mucosa associated lymphoid tissue
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
NFILS	Nuclear factor IL-3 induced
NHL	non-Hodgkin`s lymphoma
NK	Natural killer
NKP	Natural killer precursor
NKT	Natural killer T
PAMP	Pathogen-associated molecular pattern
PB	Peripheral blood
PCA	Principle component analyses
PD-1	Programmed cell death 1
PRR	Pattern recognition receptor
RANK	Receptor activator of NF-κB
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RUNX3	Runt related transcription factor 3
scRNA	Single cell RNA
SLO	Secondary lymphoid organs
Ss	Single stranded
TCF-1	T cell factor 1
TCR	T cell receptor
TF	Transcription factor
T_H	T-helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TOX	Thymocyte selection-associated high mobility group box protein
VIP	Vasoactive intestinal peptide
VIPR2	Vasoactive intestinal peptide receptor type 2

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7 Eidesstattliche Erklärung

Ich versichere an Eides statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis“ an der Heinrich-Heine-Universität erstellt worden ist.
Ich erkläre gleichzeitig, dass ich die vorgelegte Dissertation in dieser oder in einer ähnlichen Form noch bei keiner anderen Institution eingereicht habe. Weiterhin erkläre ich hiermit, dass vorher keine erfolglosen Promotionsversuche unternommen wurden.

Düsseldorf, den

Sabrina Bianca Bennstein