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STAGES AND TRANSCRIPTIONAL REGULATION OF INNATE LYMPHOID CELL DEVELOPMENT

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LIST OF ABBREVIATIONS

αLP $\alpha 4\beta 7$ Expressing Lymphoid Progenitor

AhR Aryl hydrocarbon Receptor

Arg1 Arginase 1

BMP Bone Morphogenic Protein

CHILP Common Helper Innate Lymphoid Precursor

CLP Common Lymphoid Progenitor

cNK Conventional Natural Killer Cell

DP Thymocytes Double Positive Thymocytes

EILP Early Innate Lymphoid Precursor

ETP Early T Lineage Progenitors

ftILCP fetal transitional Innate Lymphoid Cell Precursor

HIV Human Immunodeficiency Virus

HMG box High-Hobiligy-Group Box

HSC Hematopoietic Stem Cell

Id2 Inhibitor of DNA Binding 2

ILC Innate Lymphoid Cell

ILC2P Innate Lymphoid Cell 2 Precursor

ILCP Innate Lymphoid Cell Precursor

LTi Lymphoid Tissue Inducer Cell

LTiP Lymphoid Tissue Inducer Cell Precursor

mRNA messenger Ribonucleoic Acid

NCR Natural Cytotoxicity Receptor

NKT Natural Killer T Cell

PLZF Promyelocytic Leukaemia Zinc Finger

pre-NKP Pre-Natural Killer Precursor

RA Retinoic Acid

RAR Retinoic Acid Receptor

rNKP refined Natural Killer Precursor

SCID Severe Combined Immunodeficiency

SP Single Positive

T_H T Helper Cell

TSLP Thymic Stromal Lymphocpoietin

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ABSTRACT

In recent years it has become apparent that innate lymphoid cells (ILCs) play major roles in infectious, inflammatory and allergic diseases as well as tissue homeostasis. However many of the models used to study the requirement for ILCs, also have effects on other cells of the immune system, particularly T cells. This deficit in ILC-specific deletion models, highlights a need to better understand ILC development for the identification of ILC specific master regulators. Our lab has recently identified a committed ILC precursor in the bone marrow and fetal liver based on the high expression of the NKT master regulator PLZF. This precursor gives rise to all known ILC lineages, ILC1, 2, and 3, but not LTi or NK. However the stages of development and factors involved in the emergence of the PLZF expressing precursor and factors involved in the trifurcation of the ILC precursor into ILC1, 2, and 3 remain to be identified. We use single-cell multiplex qPCR of defined ILC precursors to assess their transcriptional profiles and establish a hierarchy of ILC development and ILC developmental transcription factors. We identified early clusters of precursors that corresponded to αLP that expressed *Id2*, *Nfil3* and *Tox*, but lacked expression of ILC lineage specific markers. We also identified a transitional cluster of precursors that marked the bifurcation of ILCP and LTiP and interestingly expressed high levels of *Sox4* and *Runx1*, which heretofore have not been well studied in ILC development. We also identified the ILCP as the stage of lineage trifurcation of ILCs, a process which we show to occur through multi-lineage priming. These results are the basis for a blueprint of ILC development and provide insight into the differentiation of ILC lineages. The precise definition of ILC developmental precursors and intermediates will allow for the identification of novel regulators and the development of improved models to study the function of ILCs and their importance in human disease.

CHAPTER 1

INTRODUCTION

1.1 Innate Lymphoid Cells

The human body is constantly exposed to microbes and viruses that have the potential to harm us. In order to battle such pathogens, we have evolved an intricate network of cells and molecular signals, the immune system, that is absolutely essential for our survival. The two major arms of the immune system are the adaptive and the innate immune system. Whereas cells of the adaptive immune system have the capacity to develop memory to specific pathogens through the rearrangement of antigen receptors, the cells of the innate system function to sense pathogens and induce inflammation and the priming of the adaptive immune system.

Although many cells of the innate immune system have been known for a long time, the last decade revealed a new group of cells, present in both mice and humans, that had been overlooked previously and was termed Innate Lymphoid Cells (ILCs). ILCs are predominantly found at barrier tissues, such as the skin, lung and intestine, but can also be found in adipose tissue, salivary glands and the liver. Several different ILC types have been identified, that show strong correlations with T helper effector types in their cytokine and transcription factor expression profiles[1], but unlike T helper cells, ILCs have set effector programs, allowing ILCs to respond at the earliest stages of infections[2]. ILCs have gained much attention due to their function in tissue homeostasis, autoimmunity and asthma, protection from disease as well as their potential role in the acceleration of the development of colon cancer[1].

However studies about the function of specific ILC types are complicated by the lack of adequate models to test their requirement, highlighting the necessity to study ILC development and identify the regulators of ILC development and the differentiation of specific

subsets.

1.2 ILC Types

Unlike many other innate immune cells, ILCs are derived from common lymphoid progenitors (CLPs)[3,4], but do not rearrange antigen receptors and therefore are distinct from cells of the adaptive arm of the immune system.

In a bid to consolidate the nomenclature in the field that was used to describe these innate lymphoid cells, ILCs were grouped into three groups, type 1, type 2 and type 3 ILCs, based on the expression of defining transcription factors and cytokines[5].

Type 1 ILCs include conventional natural killer cells (cNKs) and ILC1, which are similar in their expression of T-bet and the production of IFNγ. cNK and ILC1 share many phenotypic characteristics as well as a common effector program. A distinguishing marker between cNK and ILC1 in the liver is the transcription factor Eomes, which is exclusively expressed in circulating cNK, but not in the tissue-resident ILC1. Originally it was thought that Eomes$^-$ CD3ϵ^-NK1.1$^+$ cells in the liver would be precursors to cNK, however CD3ϵ^-NK1.1$^+$Eomes$^-$ cells do not convert to Eomes$^+$ cNK in culture and Eomes$^-$ ILC1 depend on T-bet while Eomes$^+$ cNK do not[6–9]. Eomes in fact is sufficient to impart the cNK phenotype on ILC1 if overexpressed[10]. Other markers that distinguish cNK from ILC1 are CD49a (integrin α_1 subunit of $\alpha_1\beta_1$) and Trail on ILC1s and DX5 (clone name for CD49b or integrin α_2 subunit of $\alpha_2\beta_1$) on cNK[9]. Unlike cNK, ILC1 are thought to be not cytotoxic, with low granzyme B and perforin expression and little evidence of degranulation in in vitro assays[11,12]. cNK also circulate in the body, whereas ILC1 are tissue resident[13].

Interestingly cNK are only sufficiently produced after birth, while ILC1 predominate in the fetus[14]. Although cNK precursors in the bone marrow have been described previously[15,16], it has become apparent that both the pre-NKP (pre Natural Killer Precursor; Lin$^-$Ly6D$^-$CD244$^+$CD27$^+$IL-7Rα^+Flt3$^-$CD122$^{\text{int}}$) and the rNKP (refined NKP; Lin$^-$

$Ly6D^{-}CD244^{+}CD27^{+}IL\text{-}7R\alpha^{+}Flt3^{-}CD122^{+}$) in the bone marrow have a history of PLZF (Promyelocytic leukaemia zinc finger)expression, which is an exclusive marker for an ILC1, 2 and 3 precursor. Due to the similarity in expression profiles it is therefore hard to distinguish ILC1 from cNK precursors and it remains to be determined what the last common precursor between cNK and ILC1 is.

Type 2 ILCs are composed of ILC2 only, which depend on the transcription factors Gata3, RORα and Bcl11b. ILC2s are innate sources of IL-5, IL-13 and the growth factor amphiregulin. ILC2 can be found at a range of barrier tissues, like the gut, lung, skin and interestingly also in adipose tissue lining the intestine[17,18]. The production of T_H2 cytokines by ILC2 in response to IL-33 and IL-25 signaling has been shown to be protective in helminth infections in the absence of the adaptive immune system and leads to eosinophilia in models of lung inflammation[19–22]. ILC2s can also be stimulated by TSLP (Thymic Stromal Lymphocpoietin) in the skin and lung, where TSLP mediated activation of ILC2s and in fact exacerbated the disease phenotype in models of skin inflammation[23–25]. ILC2 interestingly also function in fat homeostasis, whereby ILC2 promote the beiging of fat, leading to higher energy consumption[26,27]. Loss of ILC2 and ILC2 mediated IL-4R signaling in these tissues is causative of obesity. Interestingly ILC1 also play a role in obesity, where IFN-γ production by ILC1 can lead to hyperglycemia and diabetes in mice[28].

Type 3 ILCs are a heterogenous population that like T_H17 depend on RORγt expression and produce IL-17 and especially IL-22, a cytokine that exclusively acts on non-lymphoid cells to promote healing and production of antimicrobial peptides[2,29,30]. At steady state ILC3 appear to be the predominant producers of IL-22, highlighting their importance in tissue homeostasis of the gut[29,31].

ILC3 come in a variety of different flavors, Lymphoid Tissue Inducer Cells (LTis), NCR^{+} (Natural Cytotoxicity Receptor) and NCR^{-} ILC3s.

LTis are essential for the formation of lymph nodes, Peyer's Patches and Isolated Lym-

phoid Follicles[32], where interactions of LTi and stromal cells via lymphotoxin receptors, primes stromal cells in the developing fetal tissue to attract lymphocytes and form the structures of secondary lymphoid tissues. In the mouse LTi come in two flavors, $CD4^+$ and $CD4^-$. Whether there are any functional differences between $CD4^+$ and $CD4^-$ LTi is not well understood. LTi can be distinguished from other ILC3 types by the expression of the chemokine receptor CCR6[33].

NCR^+ ILC3s, which are marked by the expression of the NK receptor NKp46[12,30], have been shown to differentiate from NCR^- ILC3s by the upregulation of T-bet expression[34,35]. This process is specific to NCR^- ILC3s and does not appear to occur in LTis, which are a stable population in culture. Once NCR^- ILC3s have acquired NCR and T-bet expression, they in fact can entirely lose the ILC3 phenotype by downregulation of RORγt, converting NCR^+ ILC3 into ILC1. Interestingly the appearance of NCR^+ ILC3s only occurs after birth upon the colonization with commensal bacteria[36,37], reflecting the need for higher production of antimicrobial peptides and therefore ILC3 mediated IL-22 signaling and barrier maintenance[38]. It was suggested that the conversion of NCR^- to NCR^+ ILC3s also depends on the level of Gata3 expression in ILC3s[39]. Gata3 mediated repression of RORγt is what allows for the conversion of NCR^- to NCR^+ ILC3s. However it is not clear what signals lead to a shift in the balance of RORγt and Gata3 in ILC3s. In addition to Gata3, Notch signaling in NCR^- ILC3s appears to be a requirement for T-bet expression and the efficient generation of NCR^+ ILC3s[40].

Although ILC3s are very diverse, there is evidence that the phenotypic diversity does not necessarily translate to functional diversity and that NCR^+ILC3 appear to be dispensable in citrobacter rodentium infections in the presence of other ILC3 types[41], which are likely to compensate for the loss of NCR^+ILC3. However a functional deficiency NCR^+ILC3 has been shown to impair the clearance of C.rodentium[42].

Plasticity in ILCs had been shown to occur for NCR^- and NCR^+ ILC3s, but until

recently it was not known whether other ILC types also could exhibit plasticity in vivo. Upon stimulation, ILC2s can be distinguished based on intermediate and high KLRG-1 expression[43]. KLRG-1^{hi} ILC2 are induced by IL-25 stimulation and can develop an ILC3 phenotype. Some of the KLRG-1^{hi} ILC2 cells also express RORγt and have the capacity to produce IL-17. It is however not clear whether bona fide ILC2 are the precursors for KLRG-1^{hi} ILC2. It was not definitively shown that KLRG-1^{hi} ILC2 are generated by KLRG-1 upregulation and ST2 downregulation after stimulation. Stimulation of ILC2 with IL-1β and IL-12, leads to the upregulation of T-bet and IFN-γ production in ILC2s[44]. This process occurred through priming of ILC2s by IL-1β to become IL-12-responsive and lose Gata3 expression.

Studies on human ILCs have also shown that a subset of ILC1s can upregulate RORγt and become ILC3s. It was suggested that these ILC1s are in fact NCR^+ ILC3s that upregulated T-bet expression and converted to ILC1s (ex-ILC3s)[45]. This conversion back to ILC3s, is dependent on an optimal cytokine milieu (IL-1β and IL-23) and the vitamin A metabolite retinoic acid (RA), which has been shown to influence the balance between ILC3 and ILC2 and which is discussed below[46].

Although ILCs have been shown to play many roles in tissue homeostasis and diseases, it is not clear whether they are strictly required in the presence of a fully functioning immune system, due to the lack of tools to specifically ablate ILCs or their specific subsets, while keeping the rest of the immune system intact. An interesting hint towards addressing the fundamental question of whether ILCs are required for protection from infections, came from a study of human patients with immunodeficiencies[47]. Stem cell transplants for SCID (Severe combined immunodeficiency) patients showed that although B, T, cNK and ILC1 cells were reconstituted normally in these patients, ILC2 and ILC3 could not always be recovered. Reconstituted SCID patients did not show a higher propensity to contract in particular common herpes virus infections, suggesting that the loss of ILCs can most likely

be overcome by redundant mechanisms of other cell types, including $\gamma\delta$T cells and $T_H$cells. However this finding does not preclude that in their presence, ILCs are not protective during early stages of infection.

Bone Marrow reconstitution studies in mice have shown that ILCs can be generated from donor bone marrow and many bone marrow precursors have been identified that are described below. However recent studies have shown that ILCs are predominantly tissue resident and that peripheral ILCs can be replenished by self-renewal in the tissues. Modest exchange of ILCs between tissues only takes place in models of chronic inflammation[13,48]. This finding is corroborated by the study of human SCID patients described above and a study of HIV (Human Immunodeficiency Virus)-infected patients, which irreversibly lose peripheral ILCs[49]. ILCs in HIV infected patients could only be maintained if viral proliferation was repressed early in infection, presumably preserving the peripheral pool of ILCs required for the repopulation of the tissues. It therefore seems likely that adult tissues are first seeded with ILCs derived from fetal liver precursors during embryonic development and that they are then maintained locally throughout adult life, although bone marrow output of ILCs persists.

1.3 ILC Precursors

Many studies have focused on peripheral ILCs and their functions in a host of varying diseases. However studying the function of ILCs has been difficult due to the lack of a system to specifically knock out only ILCs or specific ILC subsets. Therefore there is a great interest in determining ILC lineage specific developmental master regulators.

Recently several bone marrow or fetal liver Precursors to ILCs have been described. These precursors vary in their lineage potential as well as their expression profiles and it is an ongoing effort of our group to consolidate the existing data and develop a precise map of ILC development[50].

The earliest studies of ILC precursors focused on ILC3 and LTi precursors, based on their common requirement for the RAR-related orphan receptor RORγt. An early study showed that the accumulation of RORγt-dependent cells was required for the formation of lymph node anlagen in the fetus[51]. These RORγt-dependent LTis were also dependent on the inhibitor of E-proteins, Id2 (Inhibitor of DNA binding 2)and express the gut homing integrin $\alpha4\beta7$, which led to the discovery of an $\alpha4\beta7$ and IL-7Rα expressing precursor in the fetal liver. A subset of lineage negative (B220$^-$CD3ϵ^-CD11b$^-$CD11c$^-$CD19$^-$GR-1$^-$NK1.1$^-$TCRβ^-Ter119$^-$), $\alpha4\beta7^+$IL7-Rα^+ fetal liver precursors expresses intermediate to high levels or RORγt and gives rise to LTi as well as ILC3[52,53]. Interestingly this Lin$^-$IL7-R$\alpha^+\alpha4\beta7^+$ precursor lost B cell an granulocyte-macrophage-generating potential in culture compared to Lin$^-$IL7-R$\alpha^+\alpha4\beta7^-$ precursors, indicating that LTi are a distinct lineage from myeloid and adaptive immune cells. Lin$^-$IL7-R$\alpha^+\alpha4\beta7^+$ cells however do retain a T cell-generating potential if cultured under conditions where high levels of Notch signaling is available[54]. This potential is lost upon further differentiation with the upregulation of ILC lineage-defining markers[55]. Two further studies identified the upregulation of CXCR6 or CXCR5 concomitantly with RORγt on Lin$^-\alpha4\beta7^+$IL7-Rα^+ as markers of commitment to the ILC3/LTi lineage[54,56]. Since ILC3 and LTi share many developmental characteristics it was assumed that they derive from a common type 3 ILC precursor. The same assumption was made for cNK and ILC1. However, the further study of ILC precursors revealed that this model of ILC development does not hold true. In fact we now know that LTi and cNK are separate lineages from ILC1, 2 and 3, showing that different ILC types converge functionally upon maturation and have different precursors.

1.3.1 The Innate Lymphoid Cell Precursor

The first proof that ILC1, 2, 3, LTi and cNK are in fact derived from developmentally distinct precursors, came from a study by our group, in which we investigated the role of the natural

Table 1.1: Staining profiles of different ILC precursors.

Progenitor	Staining Profile	Reference
αLP	$Lin^{-}CD127^{+}\alpha4\beta7^{+}CXCR5^{-}PLZF^{-}TCF\text{-}1^{-}$	55,57
EILP	$Lin^{-}CD127^{-}\alpha4\beta7^{+}Sca\text{-}1^{low}cKit^{+}Flt3^{-}Thy1^{-}CD25^{-}$ $CD122^{-}CXCR6^{-}TCF\text{-}1^{+}$	57
$CXCR6^{+}\alpha$LP	$Lin^{-}CD127^{+}\alpha4\beta7^{+}Sca\text{-}1^{low}cKit^{+}Flt3^{-}CXCR6^{+}$	58
CHILP	$Lin^{-}CD127^{+}\alpha4\beta7^{+}cKit^{+}Flt3^{-}CD25^{-}CD27^{+}CD244^{+}Id2^{+}$	59
ILCP	$Lin^{-}CD127^{+}\alpha4\beta7^{+}Sca\text{-}1^{low}cKit^{+}CD27^{+}CD244^{+}$ $Thy1^{hi}PLZF^{+}$	60
LTiP	$Lin^{-}CD127^{+}\alpha4\beta7^{+}cKit^{+}CXCR5^{+}ROR\gamma t^{+}$	55

Table is adapted from ref. 50

killer T cell (NKT) master transcription factor PLZF in ILC development[60].

Fate mapping of PLZF using a *Zbtb16* (gene coding for PLZF) reporter mouse strain, in which a GFP-Cre fusion gene is expressed under the control of the *Zbtb16* promoter, in conjunction with a Rosa26-flox-Stop-flox-YFP strain, showed that peripheral ILCs, but not LTi or cNK were marked by a history of PLZF expression, although expression of PLZF was not retained in peripheral ILCs. Of note, this tracing strategy showed about 30% YFP expression in non ILC lineages as well, which seems to be due to the early embryonic expression of PLZF, since bone marrow chimeras of non-fate mapped CLP showed no background labeling. This data suggested the presence of a PLZF-expressing precursor to ILCs and that ILCs have a distinct developmental history from LTi and cNK.

The use of a *Zbtb16* reporter mouse strain revealed the presence of a PLZF-expressing precursor in the bone marrow and fetal liver that is Lin^{-}, expresses $\alpha4\beta7$ and IL7-Rα and was termed the ILCP (Innate Lymphoid Cell Precursor) (**Table 1.1**). Transfers of ILCP mixed with congenically marked CLP into $Rag^{-/-}\gamma c^{-/-}$ lymphopenic hosts, showed that ILCP could efficiently give rise to all ILCs, but not LTi, cNK, B and T cells.

On a single-cell level the ILCP was able to produce all three types of ILCs, showing that the ILCP is a population of bona-fide precursors for all ILCs (**Table 1.2**). Interestingly

however, the ILCP gives rise to multi-potential colonies at a rather low frequency (12.2% of all colonies grown), whereas the majority of ILCP derived colonies give rise to a single specificity of ILCs, suggesting that the ILCP rapidly differentiates into single ILC lineages. A fraction of ILCPs also express ICOS, which is associated with immature bone marrow and mature ILC2s. It is therefore very likely that the ILCP is the stage of lineage trifurcation and development of ILCs with characteristics of mature cells can be observed as early as one or two days after culturing the precursor.

1.3.2 The Common Helper Innate Lymphoid Precursor

An important observation for the study of ILC precursors is, that all ILCs, including LTi and cNK at some point during their development, depend on the E-protein inhibitor Id2[17,61]. Early models of ILC development therefore included a common Id2 expressing ILC precursor. The ILCP in fact expresses Id2, but does not give rise to the two other ILC lineages, LTi and cNK, suggesting the existence of another upstream precursor with Id2 expression.

Klose and colleagues identified a precursor using an Id2 reporter mouse strain that in addition to ILC1, 2 and 3 can also give rise to LTi[59]. The common helper innate lymphoid precursor (CHILP) is $\mathrm{Lin}^{-}\alpha4\beta7^{+}\mathrm{IL\text{-}7R}\alpha^{+}\mathrm{CD25}^{-}$ and expressed high levels of Id2 (**Table 1.1**). In transfer studies the CHILP gives rise to all ILCs and LTi, but not cNK, placing it upstream of the ILCP, which lacks LTi producing potential. However this indicates that there must be another common precursor that also can give rise to cNK in addition to LTi and ILC1, 2 and 3.

Although the CHILP appears to be an early ILC precursor, it also seems to be a mixture of different precursors. The CHILP exhibits bimodal expression of PLZF, indicating that the CHILP most likely contains the ILCP. Although the CHILP did not show expression of RORγt, it is also important to note that the analysis was performed on bone marrow, which very inefficiently gives rise to LTi and shows little to no RORγt expression, compared to

fetal liver[62]. Therefore it is very likely that the expression of RORγt in the bone marrow CHILP was underestimated.

Single-cell cultures of the CHILP clearly showed that single precursors could give rise to all ILC lineages (**Table 1.2**). However in these cultures LTi and cNK cells could not be differentiated from ILC3 and ILC1 respectively, it therefore remains to be determined whether the CHILP is a bona fide precursor to all ILCs and LTi as opposed to being a mixture of ILC and LTi precursors.

1.3.3 The $\alpha4\beta7$ Expressing Precursor

Yu and colleagues identified the αLP ($\alpha4\beta7$ expressing lymphoid progenitor) as a common precursor to ILCs, LTi and cNK in the bone marrow[58]. The αLP was characterized as $Lin^{-}cKit^{lo}Sca1^{lo}$
$Flt3^{-}IL\text{-}7R\alpha^{+}\alpha4\beta7^{+}$and, unlike the CHILP, does not express PLZF (**Table 1.1**), although we could not confirm the lack of PLZF expression using this expression profile in our hands.

Expression of CXCR6 on the αLP was associated with a loss of T cell generating potential, but did not impair this precursor's ability to give rise to all ILCs, cNK and LTi in transfer studies. In single-cell cultures the αLP gives rise to colonies with multiple ILC lineages at a much higher frequency than the ILCP and the CHILP, further suggesting that it is developmentally an earlier stage than the ILCP or CHILP. LTi could not be identified in single-cell cultures, making it unclear whether the αLP is a single common precursor to LTi and ILCs (**Table 1.2**). cNK in single-cell cultures were identified based on the expression of the cNK marker Eomes, but data generated in our group suggests, that Eomes expression, as well es DX5 expression in culture is unstable and more promiscuous and can also be found on ILC1 (unpublished data). Eomes therefore is not a reliable marker in culture to identify cNK.

It remains to be determined whether the αLP on a single-cell level can give rise to a

mixture of ILC, LTi and cNK lineages, and therefore is a precursor to all ILCs.

1.3.4 The Early Innate Lymphoid Precursor

Recently an additional bone marrow precursor was identified using a $Tcf7^{GFP}$ reporter strain[57]. This precursor was termed the Early Innate Lymphoid Precursor (EILP) and was identified based on the expression of Tcf-1 among $\text{Lin}^{-}\alpha4\beta7^{+}\text{Thy1}^{-}\text{IL-7R}\alpha^{-}$ (**Table 1.1**). When transferring this precursor into $\text{Rag}^{-/-}\gamma\text{c}^{-/-}$ hosts, the EILP gave rise to all ILCs, LTi and cNK, but not T or B cells, showing that it is a common precursor to all ILC lineages, similar to the αLP. The EILP also expresses high levels of *Tox* and *Nfil3*, transcription factors that have been shown to affect all ILC lineages and that are discussed in greater

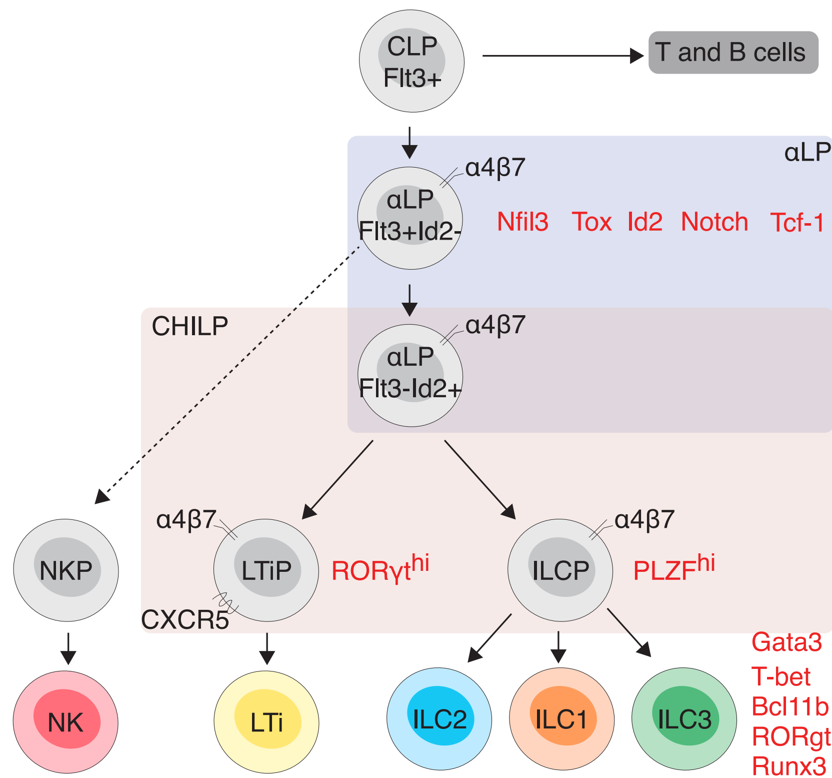

Figure 1.1: A Model for ILC development. Based on data of the developmental potential of previously described precursor and the effect of transcription factors on ILC populations, we have devised an ILC developmental schematic, in which ILCs, LTi and cNK are derived from distinct developmental precursors. Based on the published data the CHILP is represented as a mixture of $\text{Flt3}^{-}\alpha$LP, LTiP and ILCP. Transcription factors (red letters) are placed based on their relevance to individual ILC lineages.

detail below. The EILP does not express PLZF and only low levels of Id2 compared to the CHILP. While the CHILP and an early bone marrow ILC2 precursor (ILC2P) are severely affected by the lack of Id2, the EILP was only reduced by less than 1/2 in Id2 deficient mice, although the authors suggest that Id1 and Id3 are upregulated in the absence of Id2 and might compensate for its loss. These data suggest, that the EILP very likely is an early precursor to the ILCs, similar to the αLP.

Single-cell cultures of the EILP showed that a single precursor could indeed give rise to ILC1, 2 and 3, however cNK were distinguished using the marker DX5, which in culture is not reliable. Similarly LTi could not be distinguished in culture and it therefore remains to be shown whether the EILP in fact is a single precursor that can give rise to ILCs as well as cNK and LTi. However the EILP had a much greater potential to give rise to multi-potential colonies than ILCP, CHILP or αLP, showing that it indeed functions early in ILC development (**Table 1.2**).

Of note is also the lack of IL-7Rα chain expression on the EILP. Early on it had been established that the CLP can give rise to ILCs[3,4]. Consequently it was assumed that any ILC precursor after the CLP would maintain its expression of the IL-7R, which is the reason the EILP was overlooked in earlier studies. This raises the question whether the EILP represents an alternative route for ILC development or whether the EILP transiently downregulates IL-7R expression to allow for the branching of cNK, which do not express the IL-7R and don't depend on IL-7 signaling[63].

1.3.5 Arginase 1 Identifies an Intestinal ILC Precursor

Arginase 1 (Arg1)is a urea cycle enzyme that functions in the hydrolysis of L-arginine. In the periphery Arg1 was found to be expressed in ILC2, but its germline deficiency does not have major effects on ILC2 numbers or cytokine production[64]. Conditional deletion of Arg1 in the lymphoid lineage by using *Il7r*^{Cre} however showed slight defects in cytokine production

Table 1.2: Progeny of different ILC precursors in single-cell culture.

		% of colonies grown								
Precursor	Stroma	single ILC1	single ILC2	single ILC3	single LTi	Multi linage	NK	n	cloning efficiency (%)	Reference
EILP*	OP9	0.6	0	0	NR	97.6	1.8	164	50	57
CXCR6$^+$ Flt3$^-$$\alpha$LP*	OP9-DL1	1.3	5	32	NR	57.5	4.2	240	NR	58
CHILP*	OP9-DL1	13.5	28.8	11.5	NR	46.2	NR	52	NR	59
Flt3$^+$$\alpha$LP**	OP9	32.8	0	15.3	14.5	37.4	NR	135	34.6	55
	OP9-DL1†	24.5	2	8.2	0	65.3	NR	51	42.9	
Flt3$^-$$\alpha$LP**	OP9	27.4	5.5	28.8	8.2	30.2	NR	152	33.8	55
	OP9-DL1†	21.1	14.1	19.7	7	38.1	NR	77	31.4	
ILCP**	OP9	46.4	25.3	14.8	0.3	12.2	NR	925	50	55,60
	OP9-DL1†	39	24.1	16.1	2.3	18.4	NR	96	64	

NR = not reported.
* adult bone marrow progenitors, ** fetal liver progenitors.
† 36.7% of Flt3$^+$$\alpha$LP, 11.4% of Flt3$^-$$\alpha$LP and 6.8% of ILCP colonies also contained pro-T cells.
Table is adapted from ref. 50

in ILC2 that resulted in ameliorated allergic reaction to nippostrongilus brasiliensis infections. Arg1 in ILC2 supports the optimal proliferation of ILC2 by controlling amino acid metabolism and glycolysis[65].

Arg1 is expressed in the bone marrow as early as the ILC2P stage and in the fetal intestine it can be found in all ILC types[66]. It also identifies a fetal intestinal precursor to ILCs, that is Lin^{-}IL-7R$\alpha^{+}\alpha4\beta7^{+}$Arg1^{+}ROR$\gamma$t$^{\mathrm{fm}-}$ (fm for fate-mapped) NK1.1^{-}ST2^{-} (ST2 also known as IL-33Rα). These precursors interestingly coexpressed low levels of RORγt, T-bet and Gata3, suggesting a state of multilineage priming. In single-cell cultures these precursors gave rise to some dual ILC type colonies, but were not able to give rise to all ILC lineages at the same time, suggesting that the Arg1^{+} ILC precursor is developmentally after the ILCP and most cells of this population already have differentiated into specific ILC types. The Arg1 intestinal precursor most likely is a recent emigrant from the fetal liver, where ILC precursors reside before they accumulate in the bone marrow.

In the single-cell cultures of Arg1^{+} intestinal ILC precursors, LTi were not differentiated from ILC3. It is therefore not possible to determine whether this intestinal ILC precursor can give rise to LTi as well. Arg1 was also found in adult small intestinal ILC3, but whether it plays a role in ILC3 function is not clear[66].

Based on the function of Arg1 in peripheral ILC2, it is however unlikely that Arg1 plays a direct role in ILC lineage differentiation by regulating other key transcriptional regulators. Rather Arg1 most likely acts in early ILC precursors to promote their proliferation through enhancing amino acid metabolism and glycolysis.

1.4 Transcription Factors Involved in ILC Development

Recently a number of transcription factors have been shown to be involved in ILC development. Id2, Nfil3, Tox, Tcf-1 and Gata3 affect all ILCs, LTi and cNK, whereas Bcl11b, RORα, Gfi1 and PLZF only affect selected lineages. A list of transcription factors and their

effects on peripheral ILCs and ILC precursors can be found in (**Table 1.3**).

1.4.1 Id2

E proteins are a class of DNA binding proteins with a helix-loop-helix domain that facilitates homo- or heterodimerization and that have been shown to be essential for B and T cell development[67]. In B cells E2A influences the expression of the B cell lineage defining transcription factors Ebf1 and Pax5. In early T cell development E2A is required for the optimal expression of Notch1 and in the absence of E2A, HSCs develop into cNK and myeloid lineages[68].

Inhibitors of DNA binding (Id) proteins similarly have helix-loop-helix domains and heterodimerize with E proteins. Since Id proteins lack the DNA binding domain of E proteins, heterodimerization of E proteins and Id proteins leads to inhibition of E protein binding to DNA. The Id family member Id2 was very early on identified as a key factor in ILC development. Id2 knockouts lack all ILC, LTi and cNK[17,61,69].

Several studies in B and T cell development have highlighted the role of Id2 in repression of the development of adaptive immune cells. High expression levels of Id2 at the CLP stage dramatically impair the potential of CLP to give rise to T cells[70] and retroviral overexpression of Id2 in the bone marrow impairs B cell development and instead promotes erythrocyte development[71]. Similarly, overexpression of the Id2 family members Id1 or Id3 can inhibit B and T cell differentiation[72–75]. Since ILCs depend on Id2 it is conceivable that Id2 represses both B and T cell development and therefore allows for the development of ILCs. However the effect of Id2 overexpression in early progenitors on ILC development has not been formally investigated.

Interestingly cNK cells have been shown to require Id2 only at later stages of development[76], suggesting that Id2 is not required for commitment to the NK cell lineage, but more for the prevention of E protein activity and B and T cell development. However com-

pensation by other Id proteins may also explain this phenotype and it remains to be seen whether double knockouts of Id2 and Id3, which is upregulated in the absence of Id2 in cNK precursors, will show more severe defects at early cNK developmental stages.

1.4.2 Nfil3

The basic leucine zipper Nfil3 (also known as E4bp4), has been shown to function in $CD8\alpha^+$ dendritic cell development, and the circadian rhythm, where it influences the expansion of *Rorc* and thus regulates the absolute numbers of T_H17 cells in the intestine[77,78]. In ILC development Nfil3 was first shown to be required for cNK development[79,80]. However it was not clear whether Nfil3 would also be required for ILC1 development. Studies that looked at Liver ILC1, which are $CD3\epsilon^-NK1.1^+DX5^-CD49a^+$ and do not express Eomes, showed that these populations were not significantly decreased in $Nfil3^{-/-}$ mice, although there was a persisting trend towards modest reduction[9,81]. Recent studies however show that all ILC, cNK and LTi are, although not entire absent, dramatically reduced in Nfil3 knockouts, especially in competitive chimeras, suggesting that Nfil3 functions early in ILC development[82–84]. In $Nfil3^{-/-}$ mice the αLP as well as ILCP and ILC2P were drastically reduced in absolute numbers and percentage in the bone marrow and fetal liver[58,84]. Deletion of *Nfil3* in the periphery using a conditional *Nfil3* knockout in conjunction with *Rorc*^{Cre}, showed that Nfil3 was not required for the survival and maintenance of mature ILC3s in the periphery, suggesting that Nfil3 is only required early in ILC development[84].

These reports suggest that Nfil3 plays a significant in the development of all CLP-derived ILC groups, but perhaps may not be required for their survival as mature cells. ChIP of Nfil3 in transduced cell lines or bone marrow CHILP revealed that Nfil3 is capable of binding directly to the promoters of both Id2 and Eomes, suggesting that Nfil3 acts upstream of Id2 in ILC, cNK and LTi development[84,85]. However the effect of *Id2* deficiency on ILCs is much more severe than the loss of *Nfil3*, suggesting that Nfil3 is not the sole regulator of

Id2.

1.4.3 Tox

Tox belongs to the high-mobiligy-group box (HMG box) containing protein superfamily of transcriptional regulators and its conditional deletion in T cells leads to an early T cell developmental defect with a block at the DP stage[86]. Further studies showed that *Tox*-deficient mice also had defects in the development of Peyers patches, indicating a defect in LTi development[87]. *Tox* deficient mice also lacked DX5^{+} cNK cells and it was shown that in fact all ILCs as well as LTi depend on Tox for their development[88]. In the absence of *Tox*, bone marrow precursors to ILCs, the CHILP as well as a dedicated precursor to ILC2s, the ILC2P[89], were severely reduced in absolute numbers. Although Tox expression is upregulated early during ILC development, its expression is maintained in mature cells, suggesting a function for Tox in ILC maintenance.

Yu and colleagues suggested based on array and ChIP studies, that Nfil3 might act through the transcription factor Tox in innate lymphocyte development to regulate Id2[58]. In *Nfil3* knockouts Tox transcript levels were markedly reduced and retroviral overexpression of Tox in hematopoietic progenitors of Nfil3 knockout mice partially restored the defects in innate lymphocyte development.

Aside from Tox, the Tox family also includes, Tox2, Tox3 and Tox4, and Tox2 has been suggested to play a role in human cNK development by influencing T-bet expression[90]. However, not much is know about the function of these three transcription factors, especially in regard to ILC development.

1.4.4 T cell factor 1

Tcf-1 (encoded by *Tcf7*) is known to act in concert with Lef-1 and β-catenin in T cell development. *Tcf7* is expressed as early as the ETP (Early T lineage progenitor)stage and

Tcf7$^{-/-}$ knockout mice have severe defects in early T cell development[91–93]. At the DP stage Tcf-1 and Lef-1 regulate the expression of Th-POK and in their absence $CD4^+$ T cell development is dramatically impaired[94]. Interestingly Tcf-1 is regulated by Notch signaling and represses Id2 in ETP[93]. Tcf-1 has also been shown to regulate the expression of *Gata3* and *Bcl11b*[92].

Tcf-1 was first shown to play an important role in ILC2 development, but, with the discovery of other ILC lineages, its function in ILC development was rapidly expanded to include all ILC types. *Tcf7*-knockout mice have defects in ILC2, the NCR^+ subset of ILC3, cNK and ILC1[95,96]. In competitive chimeras however the defect in ILCs is much more severe, affecting all known ILC subsets[57]. This discrepancy can be explained by the dual role Tcf-1 might play in ILCs. Tcf-1 clearly plays a role in early ILC development and is required for the differentiation of all ILC subsets, however *Tcf7*$^{-/-}$ ILC3s show higher expression levels of RORγt and a greater capacity to produce IL-17 and IL-22, showing that in mature ILCs Tcf-1 represses the ILC3 fate[97]. It has been proposed that Notch signaling regulates *Tcf7* expression in ILCs, however deletion of the Notch DNA binding partner RBP-J_κ does not alter *Tcf7* expression[98].

1.4.5 Gata3

The GATA (GATA-binding protein) family member Gata3 is widely expressed during development and in adult tissues, such as the central nervous system, adrenal glands, kidneys and others. In the hematopoietic system GATA3 is best known for its role in T helper (T_H) cell differentiation, where it directs the development of T_H2 effector cells by repressing the T_H1 and T_H17 programs[99,100]. Its function in T cell development is much broader, where at the DP stage it also regulates the expression of TCR subunits, ThPOK and Notch1. Depending on the cell type Gata3 can associate with multiple cofactors to effect a cell specific expression profile[100].

Gata3 was first shown to be important for ILC2 development and maintenance, but recently it has been recognized to play a broader role in the development of all ILC lineages[89,101,102]. Yagi and colleagues showed a dramatic reduction of ILC1, 2, 3 and LTi in $Vav^{Cre}Gata3^{fl/fl}$ mice, cNK however were unaffected[102]. The authors also showed that the early $Lin^{-}IL\text{-}7R\alpha^{hi}Flt3^{-}\alpha4\beta7^{+}$ fetal liver precursor was absent in *Gata3*-deleted animals, suggesting that lymphoid tissue inducer precursors (LTiP) and ILCP which have higher IL-7Rα expression were predominantly affected. In contrast to Nfil3, which affects all ILC, LTi and cNK, Gata3 appears to act later during ILC development, placing it downstream of Nfil3, after the branching of cNK, to promote LTi and ILC development. Gata3 also did not appear to affect Id2 expression levels, unlike the upstream factors Nfil3 and Tox. In fact Gata3 expression coincides precisely with the upregulation of PLZF at the ILCP stage, indicating that it acts late in ILC development[60].

1.4.6 PLZF

The transcription factor PLZF has been shown to be a master regulator of NKT development and defines the ILCP[60,103]. Although PLZF is expressed at the ILCP stage, its deletion predominantly affects only ILC2 and ILC1 but not ILC3[60]. Loss of *Zbtb16* both reduces the total numbers of ILC2, but also affects their function during airway inflammation in the presence of a functioning adaptive T_H2 response[104]. PLZF in fact directly regulates factors involved in ILC2 lineage specification, such as *Rorc*, *Runx1*, *Rora*, *Icos*, *Il4* and *Il13* in NKT cells[105]. Interestingly PLZF also represses the expression of *Bach2*, which has been shown to repress T effector programs in favor of a T regulatory program in T cells[106]. It is very likely that PLZF fulfills a similar role during ILC development to promote ILC effector program establishment by repressing Bach2 and promoting the ILC effector programs.

1.4.7 Ets1

The family of Ets transcription factors has been shown to regulate Id2 expression. *Ets1*-knockout mice show defects in cNK, where Ets1 regulates *Tbx21* and *Id2* expression[107]. Until recently it was not known whether Ets1 also plays a role in other ILC lineages. In conditional *Ets1*-knockout mice, where *Ets1* was deleted by crossing *Ets1*-floxed mice to $Il7r^{Cre}$ mice, peripheral ILC2 were dramatically reduced in numbers[108]. However the early ILC precursor, CHILP, was unaffected in numbers in these mice. The *Ets1*-deficient CHILP however had a reduced potential to give rise to ILC2s, showing that Ets1 acts early in ILC2 differentiation. Although Ets1 has been shown to play a role in cNK development and function, these studies have not distinguished between cNK and ILC1. It is therefore not clear whether Ets1 also plays a role in ILC1 development.

1.4.8 Runx3

The runt-related transcription factors (Runx) play major roles in hematopoiesis and the roles of Runx1, Runx3 and Th-POK have been well studied in T-cell development[109]. Runx3 and Th-POK play an important role in the differentiation of CD4 and CD8 single positive (SP)thymocytes, where Runx3 promotes the development of CD8SP T cells in the thymus, by inhibiting the CD4SP T cell master transcription factor Th-POK[110].

Runx3 has been shown to be expressed in ILC1 and ILC3 and its expression in the fetal liver and bone marrow Lin^{-}IL-7R$\alpha^{+}\alpha4\beta7^{+}$ precursors overlaps with PLZF expression, showing that Runx3 acts at the ILCP stage and most likely in the lineage differentiation of ILC1, 2 and 3[111]. In fact Runx3 expressing ILCP predominantly give rise to ILC1 and ILC3, but not ILC2, highlighting the close relationship of these two ILC lineages, which has been established by evidence of ILC3 interconversion to ILC1 in the periphery.It is however not clear whether the conversion of ILC3 into ILC1 is the default developmental pathway of

Table 1.3: Consequences of genetic ablation of transcription factors on the development of ILC, LTi and cNK.

	Bone Marrow					Periphery					
Knockout	αLP†	EILP	CHILP	ILCP	ILC2	ILC1	ILC2	ILC3	LTi	cNK	References
Id2	↓↓	↓	NR	NR	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	51,61,76,89 17,57,63,112
Nfil3	↓↓↓	NR	↓↓↓	↓↓↓	↓↓↓	↓ − ↓↓↓	↓↓↓	↓ − ↓↓↓	↓ − ↓↓↓	→ / ↓↓↓	58,82–84 9,79,80,113
Tox	↓↓↓	NR	↓↓↓	NR	↓↓↓	↓↓↓	↓↓↓	↓↓	↓↓↓	↓↓↓	87,88
Tcf7	NR	NR	↓↓↓	↓↓↓*	↓↓↓	→/ ↓↓↓*	↓↓↓	NCR+ ↓↓/ ↓↓↓*	↓/ ↓↓↓*	→/ ↓↓↓*	57,95,96,114
Zbtb16	NR	NR	NR	NR	↓*	IEL →* Liver ↓↓*	↓↓*	→	→	→	60
Rora	NR	NR	NR	NR	↓↓	NR	↓↓*	→	NR	→	25,115
Gata3	↓↓ IL7Rα^{hi}	NR	NR	NR	↓↓↓	↓	↓↓↓	↓↓↓	↓↓↓	→	101,102,116
Runx3	→	NR	→	→	→	↓	→	↓↓↓	↓↓↓	↓↓↓	111
Bcl11b	NR	NR	NR	NR	→/ ↓↓↓	→	↓↓↓*/ ↑	→/ ↑*	NR	NR	117–119
Gfi1	NR	NR	NR	NR	↓↓↓	NR	↓↓	NR	NR	NR	120

† Here αLP covers all $Lin^{-}CD127^{+}\alpha4\beta7^{+}$ cells.
* Phenotype found in mixed chimeras only.
NR = not reported.
→ no change in population.
↓ or ↑ 1.3-3 fold change in population.
↓↓ or ↑↑ 3-5 fold change in population.
↓↓↓ or ↑↑↑ more than 5 fold change in population.
Table is adapted from ref. 50

ILC1 or whether there exist two independent precursors in the bone marrow and fetal liver to ILC1 and ILC3.

1.4.9 ILC2 Lineage-Defining Transcription Factors

Several transcription factors have been associated specifically with the development of ILC2s. Gfi-1 and RORα were the first transcription factors that were identified to act solely on ILC2s. In both RORα mutant and *Gfi1* knockout mice, both peripheral ILC2s as well as bone marrow ILC2Ps were dramatically reduced in number[25,115,120]. Functionally *Gfi1*$^{-/-}$ ILC2s were also impaired in their response to IL-33 stimulation. Similarly RORα mutant ILC2s showed impaired expansion in response to challenge[25,115]. *Gfi1*$^{-/-}$ ILC2s also acquire the capacity to produce the ILC3 cytokine IL-17 and upregulation of *Rorc*, suggesting that Gfi1 enforces the ILC2 effector program, by inducing the expression of Gata3 and other ILC2-specific factors and repressing the ILC3 gene module.

In T cells Bcl11b has been shown to be required for the commitment to the T lineage. In the absence of *Bcl11b* T cell precursors can be redirected to the cNK lineage[121,122]. This change appeared to be affected mainly by the loss of Notch and Notch target gene expression in *Bcl11b* deficient mice. Bcl11b also regulates the expression of Foxp3 in CD4 single positive T cells, and its loss in CD4^{+} T cells leads to a loss of regulatory T cells[123].

In ILCs, Bcl11b can exclusively be found in ILC2s in the periphery and at the CHILP stage in the bone marrow, where it overlaps with the expression of ICOS, an ILC2 specific marker. In in vitro cultures Bcl11b^{+}CHILP were restricted to give rise to ILC2[118]. Defects in ILC2 numbers in *Bcl11b*-deficient mice were mostly apparent in competitive bone marrow chimeras, suggesting that loss of Bcl11b potentially can be compensated for by other ILC2 speci‘’fic transcription factors. Although *Bcl11b*$^{-/-}$ mice show mild defects in ILC2s, the remaining ILC2 show functional defects upon challenge. Interestingly conditional deletion of *Bcl11b* in mature ILC2s also effects their conversion into ILC3, with the upregulation of

RORγt and loss of Gata3 expression and the aquisition of IL-23 responsiveness and IL-22 and IL-17 production[119]. This loss of the ILC2 program could be overcome by overexpression of Gfi1, which is directly controlled by Bcl11b. Single-cell RNA sequencing studies showed that the block in ILC2 development in *Bcl11b*$^{-/-}$ bone marrow precursors occurred at the ILCP stage, when *Bcl11b* was upregulated[124]. ILC2P could not be found in these mice.

1.5 Other Factors Involved in ILC Development

Aside from transcription factors, extracellular cues such as cell to cell contact signaling as well as cytokine and environmental antigen signaling have been shown to influence ILC homeostasis and development.

1.5.1 Notch Signaling

Notch signaling plays a seminal role in many developmental processes of the hematopoietic system. Notch has been suggested to function in hematopoietic stem cell (HSC) self-renewal and maintenance and is well known for its requirement in T-cell development[125,126].

Notch signaling was suggested to play a role in early ILC development, by augmenting the upregulation of $\alpha 4\beta 7$ on CLP in culture[56] and was also sufficient for the upregulation of RORγt in cultures of bone marrow ILC precursors, but was not required for the same process in fetal liver precursor cultures[54]. Similarly Notch signaling augmented the differentiation of ILC2 in culture of the αLP, but not the ILCP[55,57,115].

Yang and colleagues suggested that the upregulation of Tcf-1, a known Notch target, in the EILP, depends on Notch signaling, but whether this is in fact the case was not clear[57]. Studies in which the Notch DNA-binding adapter RBP-J_κ was conditionally deleted using *Il7r*^{Cre} mice showed no change in the expression of *Tcf7* in ILC precursors in the fetal liver[98]. In fact overexpression of Notch using the expression of the Notch intracellular domain in fetal livers, repressed *Tcf7* expression and promoted the development of T cells from fetal liver

CLP.

Since Notch signaling appears to be dispensable for ILC development and yet augment aspects of it, it begs the question as to the function of Notch in ILC development. In T cells, Notch signaling has been shown to directly regulate Gata3 expression as well as influence T-bet expression[127–129], thereby regulating the balance between T_H2 and T_H1 effector differentiation and maintenance. Notch signaling therefore may influence optimal Gata3 expression to promote ILC differentiation.

In conclusion Notch is not required for ILC development and it remains to be determined how *Tcf7* is regulated and promotes the emergence of the EILP.

1.5.2 Aryl Hydrocarbon Receptor and Vitamin A Signaling

ILCs are almost exclusively found in barrier tissues, such as the skin, lung and intestine. Particularly in the intestine, ILCs have been shown to also respond to dietary changes.

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that is known to bind environmental toxins (dioxins) as well as dietary ligands such as flavonoids and glucosinolates, which can be found in plants[130]. The AhR mainly controls the expression of cytochrome P450 xenobiotic-metabolizing enzymes and is expressed in ILC3 in the intestine.

Ahr-deficient mice have a reduction in ILC3 and LTi, that is already noticeable at birth and persists in adults[30,131,132]. Loss of *Ahr* in ILC3 also affects the formation of cryptopatches as well as isolated lymphoid follicles. The size of the ILC3 pool in the intestine can be regulated by AhR ligand in the diet, showing that dietary antigens can in fact have an influence on ILC populations[131]. ILC1 however were not affected in $Ahr^{-/-}$ mice[12,133].

Interestingly activation of the AhR could also upregulate Notch1 and Notch2 in intestinal lamina propria lymphocytes, but it is not clear whether the regulation of Notch by the AhR occurs in the periphery only or could potentially act in early bone marrow ILC precursors[133].

Gene regulation by the AhR can influence the metabolism of the Vitamin A metabolite RA[134], which has been shown to play essential roles during embryonic development, organogenesis and influences oral tolerance through the production of TGF-β and differentiation of induced T regulatory cells[135,136]. RA can augment T effector responses during infections and leads to the upregulation of the gut homing integrin $\alpha 4 \beta 7$ and the chemokine receptor CCR9 on both T cells and ILCs[137,138].

Spencer and colleagues showed that RA can influence the balance between ILC2 and ILC3, favoring the expansion and functional augmentation of ILC3, whereas vitamin A deficiency resulted in an expansion of ILC2[46,139]. As a consequence vitamin A-deficient mice had an impaired response to Citrobacter rodentium infections, which at early time points require an ILC3 response with IL-22 production for clearance. In contrast worm burden in helminth-infected vitamin A deficient mice was significantly reduced due to increased IL-13 production in the intestines of these animals. The effect of RA signaling on the balance of ILC3 and ILC2 also appears to play a role during ILC development in the fetal liver and fetal gut as maternal retinoids can affect the balance of ILC3 in fetuses by controlling *Rorc* expression[140].

Although it is apparent that dietary antigens play a role in ILC homeostasis it is not known whether either AhR or RA signaling affect early developmental stages of ILCs or whether these factors affect ILC maturation or maintenance in the periphery.

In summary, multiple transcription factors and signals that influence ILC development have been discovered, but a precise definition of the stages and the sequence of ILC development, as well as the molecular mechanisms that drive ILC development have remained elusive.

CHAPTER 2

AIMS AND SIGNIFICANCE

ILCs have been shown to play important roles in the protection against infections and their dysregulation is associated with the exacerbation of inflammation in inflammatory diseases as well as the formation colon cancer. The models of ILC deficiency used to study the requirement of ILCs in infections and inflammation heavily rely on knockouts of transcription factors also required in other immune cells, predominantly T cells. Therefore it remains difficult to study the requirement of ILCs in an otherwise immune sufficient host, highlighting a need to understand ILC development and more specifically its regulators. The identification of ILC-specific regulators will offer new strategies in determining the role of ILCs in immune sufficient hosts and potentially lead to new therapeutic strategies in the treatment of diseases, in which ILCs play important roles.

Several precursors to ILCs have been described previously, including the αLP, CHILP and ILCP, but their relationship and the precise hierarchy of transcription factors, remains elusive.

We will use a single-cell RNA approach to better characterize stages and infer transitions, which will be further investigated by transfer studies.

The aims of my studies are:

- Use the PLZF-GFPCre^{+} ILCP to define the developmental hierarchy upstream and downstream of ILC precursors.

- Identify the branch point of LTi and ILCs. To address this question we performed single-cell cultures of ILC precursors to assess their potential to give rise to ILCs as well as LTi.

- Identify the stage of lineage trifurcation where cells acquire effector programs.

- Assess whether PLZF is sufficient to drive the ILC program in early ILC progenitors. To address this question we used both retroviral and transgenic overexpression methods.
- Assess the role of Tox2, a new transcription factor upregulated at the ILCP stage.

CHAPTER 3

RESULTS

3.1 Single cell analysis defines the divergence between the innate lymphoid cell and lymphoid tissue inducer lineages

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3.1.1 Summary

The precise lineage relationship between innate lymphoid cells (ILC) and lymphoid tissue inducer (LTi) cells is poorly understood. Using single-cell multiplex transcriptional analysis of 100 lymphoid genes and single-cell cultures of fetal liver precursor cells, we identified the

common proximal precursor to these lineages and found that its bifurcation was marked by the differential induction of the transcription factors PLZF and TCF1. Acquisition of individual effector programs specific to the ILC subsets ILC1, ILC2 and ILC3 was initiated later, at the common ILC precursor stage, by transient expression of mixed ILC1, ILC2 and ILC3 transcriptional patterns, whereas, in contrast, the development of LTi cells did not go through multilineage priming. Our findings provide insight into the divergent mechanisms of the differentiation of the ILC lineage and LTi cell lineage and establish a high-resolution 'blueprint' of their development.

3.1.2 Introduction

Innate lymphocytes lack B or T cell receptors and exert effector functions at mucosal barriers[141,142]. These populations segregate into three general groups on the basis of their expression of the transcription factors T-bet, GATA-3 and RORγt. However, there is considerable heterogeneity among T-bet-expressing group 1 lymphocytes, which comprise conventional (or classical) natural killer (NK) cells (cNK cells), group 1 innate lymphoid cells (ILC1 cells) and tissue-resident NK cells, and among RORγt-expressing group 3 lymphocytes, which comprises CCR6$^+$ lymphoid tissue inducer (LTi) cells and CCR6$^-$ ILC3 cells. In addition, some plasticity has been reported among CCR6$^-$ ILC3s which can upregulate T-bet expression and acquire group 1 properties[34], and among some populations of ILC2s which can acquire group 3 properties[43].

Lineage-tracing and cell-transfer studies have suggested that ILC1, ILC2 and ILC3 cells, but not LTi cells or cNK cells, are derived from a common dedicated precursor, the ILC precursor (ILCP), characterized by expression of the transcription factor PLZF[60]. Similar to the LTi precursor (LTiP), the ILCP originates from a lymphoid precursor that expresses the integrin $\alpha4\beta7^+$ and is itself derived from the common lymphoid precursor (CLP). The Id2^{hi} fraction of $\alpha4\beta7^+$ lymphoid precursors, called 'common helper innate lymphoid precursor'

(CHILP), is a heterogeneous population that includes the PLZF-expressing ILCPs as well as precursors to LTi cells[59], but whether the CHILP population includes a common precursor of both ILCs and LTis or separate precursors of each lineage has not yet been determined. A study has suggested that cNK cells might originate from an earlier $Id2^{lo}CXCR6^{+}$ fraction of $\alpha4\beta7$-expressing lymphoid precursors (αLPs) 7. Thus, the developmental relationships between lineages remain incompletely established.

Several transcription factors, including *Nfil3*, *Tox*, *Id2*, *Gata3*, *Tcf7* and *Zbtb16* (encoded by PLZF)[17,58,60,82,84,88,89,95,96,101,102] are required for the development of all or several of these innate lineages, which suggests they have an effect at a common precursor stage. However, partial defects, rather than complete defects, have often been reported in mice lacking these transcription factors, which suggests substantial redundancy and complexity of this early transcriptional network. Other transcription factors have been found to selectively affect individual ILC lineages, such as the effect of RORα (encoded by *Rora*), Bcl-11b and Gfi1 in ILC2 cells[115,117,120], which suggests more distal effects in the ILC-differentiation pathway. Precise understanding of the general hierarchy of expression of these factors is missing, however, which limits the design and interpretation of mechanistic studies aiming at delineating their interplay.

Here we used cultures of single cells purified from the fetal livers of a mouse reporter strain expressing green fluorescent protein (GFP) and Cre recombinase from the gene encoding PLZF (*Zbtb16*-GFP-Cre) to precisely define the differentiation stages between CLP and ILC and to identify the stage of divergence between ILCs and LTi cells. We also performed multiplex quantitative single-cell transcriptional analysis of 100 lymphoid genes to characterize the complexity of molecular events associated with this differentiation pathway. We derived a high-resolution map and inferred a precise ordering of the induction of transcription factor expression and identified previously unknown stages of development before and after PLZF expression and the stage of bifurcation between the ILC lineage and LTi cell lineage.

Notably, transcriptional priming for the different cytokine effector programs occurred at the ILCP stage itself through multilineage priming. In contrast, the LTiP proceeded to directly acquire its type 3 program without undergoing mixed transcriptional priming. Together our findings further define the dichotomy between ILCs and LTi cells and provide new insight into the stages and mechanisms of their development and the interplay of transcription factors that direct their differentiation.

3.1.3 Results

3.1.3.1 Bifurcation of αLP into ILCP and LTiP

Lin$^-$IL-7Rα^+ fetal liver cells (where lineage (Lin) is defined by a 'cocktail' of antibodies to CD3ϵ, TCRβ, CD19, CD11c, GR-1, Ter119 and NK1.1), include CLPs, identified by a Flt3$^+\alpha4\beta7^-$ profile, and a cell population expressing $\alpha4\beta7$, which included the precursors to innate lymphoid lineage, thought to arise from the CLP (**Fig.3.1a**). This $\alpha4\beta7$-expressing population was originally called 'αLP', but the identification of ILCPs and LTiPs among αLPs prompted us to limit the designation of αLP to the $\alpha4\beta7$-expressing cells that were neither ILCP nor LTiP but include their precursors[142,143]. We further subcategorized this αLP as an Flt3$^+$ and Flt3$^-$ subsets to identify early and late precursors, respectively. We detected these populations by flow cytometry among fetal liver cells obtained from the *Zbtb16*-GFP-Cre reporter strain at embryonic day 15 (E15)(**Fig.3.1a**). Among the Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$ population, the ILCPs were identified by GFP expression and the LTiPs were identified by their GFP$^-$CXCR5$^+$ profile[60,112]. The LTiPs were clearly distinguishable from the other subsets in this staining, although a small fraction of these cells seemed to have low expression of GFP (**Fig.3.1a**). As expected, only the LTiP coexpressed the chemokine receptor CCR6 and the coreceptor CD4 (**Fig.3.1b**).

Both LTiPs and ILCPs could already be detected at low frequencies among Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$ fetal liver cells at E12, the earliest time point in our analysis, and their absolute

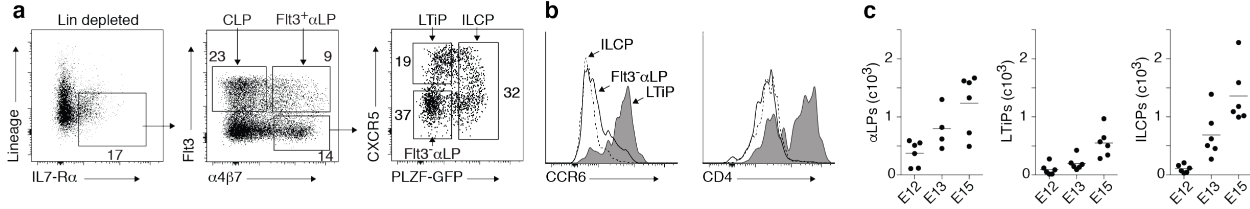

Figure 3.1: Identification of distinct subpopulations of $\alpha4\beta7$-expressing lymphoid precursors in fetal liver. (**a**) Flow cytometry of fetal liver cells from E15 *Zbtb16*-GFP-Cre reporter mice after depletion of Lin$^+$ cells (expressing CD3, CD11c, CD19, NK1.1, TCRβ, Ter119 and GR-1) by magnetic bead-based cell separation and staining for IL-7Rα^+ (left), Flt3 and $\alpha4\beta7$ (middle) and CXCR5 (right), for the identification of distinct subpopulations representing CLPs, αLPs, LTiPs and ILCPs (middle and right) after gating on LinIL-7Rα^+ cells (left). Numbers adjacent to outlined areas indicate percent cells in each subpopulation. (**b**) Flow cytometry of ILCPs, LTiPs and Flt3$^-$ αLPs stained for CCR6 or CD4. (**c**) Quantification of αLPs, LTiPs and ILCPs in fetal liver at E12, E13 and E15. Each symbol represents an individual fetus; small horizontal lines indicate the mean. Data are representative of twelve independent experiments (**a**) or three experiments (**b**) or are pooled from two independent experiments (**c**).

numbers increased five- to tenfold by E15 (**Fig.3.1c**). To establish the lineage relationships between αLP, ILCP and LTiP, we performed single-cell cultures of αLP subsets (Flt3$^+$ and Flt3$^-$) on OP9 stromal cells in the presence of interleukin 7 (IL-7) and stem cell factor (SCF), as described[60]. We categorized ILC1 colonies, ILC2 colonies and ILC3 colonies by high expression of NK cell receptor NK1.1, the costimulatory receptor ICOS or $\alpha4\beta7$, respectively, as described previously[60]. We distinguished LTi cells from ILC3 colonies by expression of CD4, which is expressed by nearly half the LTi cells but not in ILC3s (**Fig.3.1b**), although this method underestimated the real frequency of LTi cell colonies by approximately twofold. We could not distinguish cNKs from ILC1s by expression of eomesodermin (Eomes), because expression of this transcription factor was induced in ILC1 cells in our culture conditions (data not shown). However, because fetal progenitors do not produce cNKs[14], we counted all NK1.1$^+$ colonies as ILC1 cells.

ILCPs gave rise nearly exclusively to single and mixed colonies of ILC1 cells, ILC2 cells and ILC3 cells (**Fig.3.2**), consistent with this cell type's being a common precursor to these lineages, as reported[60]. In contrast, Flt3$^+$ and Flt3$^-$ αLPs also generated a sizeable proportion of LTi cells, which indicated that this group of cells included precursors to the LTi cell lineage. Notably, many wells containing LTi cells also included ILC1 cells or ILC2

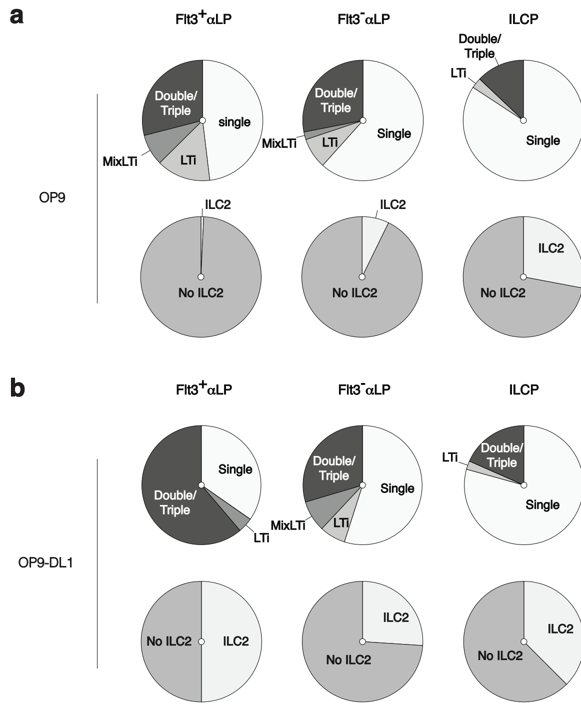

Figure 3.2: Colonies derived from single-cell cultures of $\alpha\mathbf{4}\beta\mathbf{7}$-expressing lymphoid precursors in fetal liver. Frequency of single ILC1, ILC2 or ILC3 cells (Single) or two or more ILC subsets (Double or triple), LTi cells, and mixed colonies of LTi (CD4+) cells plus either ILC1 cells or ILC2 cells (the presence of ILC3 cells could not be ascertained in these wells) (MixLTi) (top), and frequency of wells containing ILC2 colonies (whether single or mixed with other ILC lineages) (bottom), among single-cell cultures of various precursors sorted from *Zbtb16*-GFP-Cre fetal livers at E14-E16 and cultured with OP9 cells (**a**) or OP9-DL1 cells (**b**). Average cloning efficiency, 31-64%; ¡3% of colonies could not be characterized by staining and are not presented here. In OP9-DL1 cultures, colonies of pro-T cells were also observed in 47% of Flt3+ LP wells, 19.7% of Flt3$^-$ αLP wells and 14.5% of ILCP wells; these were identified by a CD4$^+$ or CD8$^+$, ICOS$^-\alpha4\beta7^-$NK1.1$^-$ phenotype (data not shown). Data are pooled from at least three independent experiments (OP9) or one to three independent experiments (OP9-DL1) with 51-447 total colonies for each progenitor population.

cells (the presence of ILC3 cells could not be ascertained in wells containing LTi cells), which indicated that a single Flt3$^+$ or Flt3$^-\alpha$LP precursor could generate both LTi cells and ILC lineages. Thus, while the reported CHILP population[59] included a heterogeneous mixture of ILCPs and other precursors, our observations identified the Flt3$^-\alpha$LP as the common proximal precursor, at the single-cell level, of both ILCPs and LTiPs.

Notably, when we performed the cultures with OP9 stromal cells lacking the DL1 ligand of Notch receptors, we found that many fewer ILC2 colonies were derived from Flt3$^+\alpha$LPs or Flt3$^-\alpha$LPs than from ILCPs (**Fig.3.2a, bottom**). However, this defect was largely corrected in OP9-DL1 cultures (**Fig.3.2b, bottom**). This finding was consistent with the proposal that a Notch signal is essential for ILC2 differentiation[96,115] and further established that the signal must be delivered early at the LP stage, rather than late at the ILCP stage. Together these experiments demonstrated that the fetal liver αLP pathway was dedicated to the formation of the ILC and LTi cell lineages. They also identified the late Flt3$^-\alpha$LP as the common proximal precursor to ILCPs and LTiPs.

3.1.3.2 Single-cell multiplex qRT-PCR analysis of ILC precursors

We used multiplex quantitative RT-PCR for transcriptional analysis of single cells along the fetal pathway linking αLPs to ILCPs and LTiPs, with a panel of 100 probes for genes encoding lymphoid development factors, including transcription factors, cytokine receptors and chemokine receptors. Data from 339 single cells, including 157 αLPs, 168 ILCPs and 14 LTiPs, were compiled from two independent single-cell sorting experiments with pooled E15 fetal livers. After filtering results by the expression of 'housekeeping' (control) genes, we considered the transcriptional profiles from 299 single cells for downstream analysis.

Unsupervised hierarchical clustering analysis of these transcriptional profiles defined

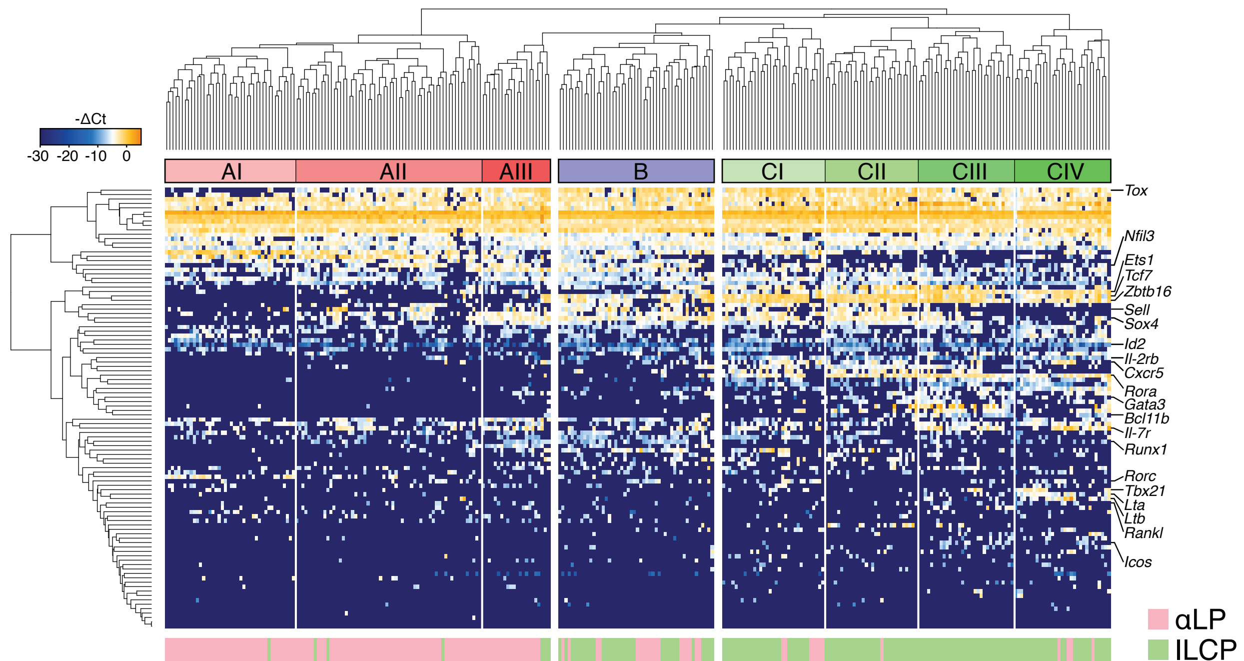

Figure 3.3: Hierarchical clustering distinguishes αLP and ILCP transcriptional profiles. Hierarchical clustering dendrogram (top and left margin) derived from single-cell multiplex quantitative PCR analysis of 299 single αLPs and ILCPs, distinguishing clusters AI-AIII (αLPs; left), cluster B (mixed αLPs and ILCPs; middle), and clusters CI-CIV (ILCPs; right); each column represents a single cell (bottom, sorted cell type; key, bottom right), and each row represents one gene (of 100 total; right margin, select genes encoding products with known or anticipated roles in ILC development). ΔCt (top left key), difference in threshold cycle relative to the average value for the 'housekeeping' (control) gene. Data are from two independent experiments with 157 αLPs and 168 ILCPs from pooled livers.

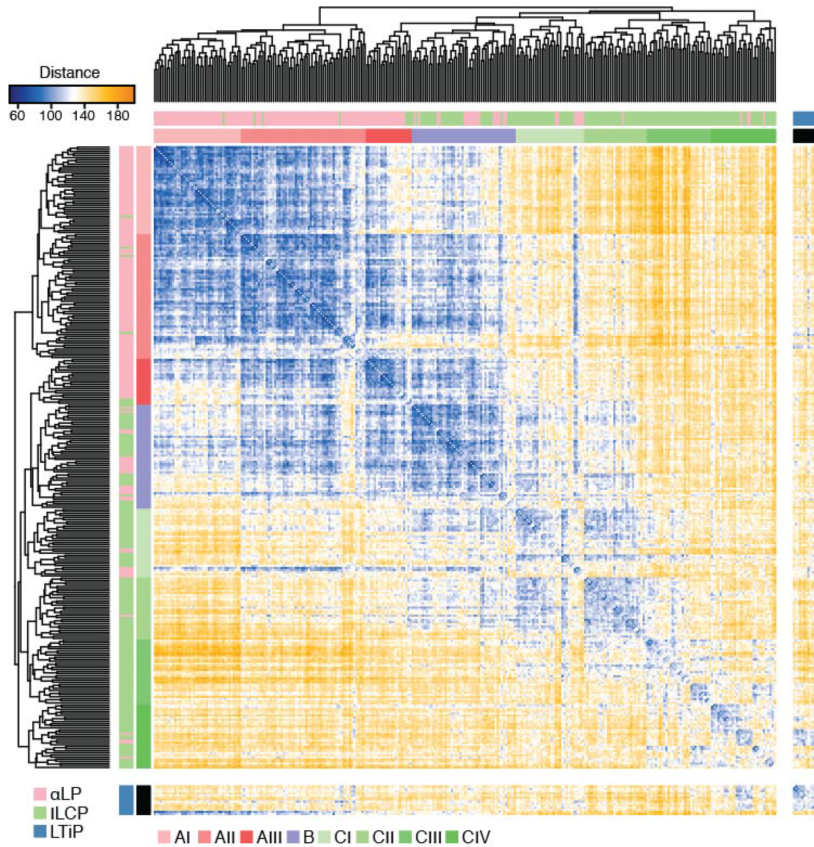

Figure 3.4: Intercellular transcriptional distances confirm hierarchical clustering analysis. Euclidean distance between all pairs of measured transcriptional profiles. Dendrograms shown are those obtained from the hierarchical clustering of these intercellular distances. Outer bar, sorted cell type; αLP (red), ILCP (green), LTiP (blue). Inner bar, cluster assignment of each cell; AI-III (red), B (purple), CI-IV (green).

groups of cells that seemed to be in distinct developmental stages (**Fig.3.3**). Consistent with the proposal that acquisition of PLZF expression signified a distinctive developmental transition for ILCs, we found strong, visually evident separation of the transcriptional profiles of the αLP populations (**Fig.3.3, left**) and ILCP populations (**Fig.3.3, right**). We used contiguous branches of the hierarchical clustering dendrogram to define three clusters of predominantly αLPs (AI-AIII), one cluster composed of mixed LPs and ILCPs (B), and four clusters of predominantly ILCPs (CI-CIV), on the basis our understanding of ILC development. All clusters were distinct from one another and were composed of a substantial number (>20) of similar cells (**Fig.3.4**). One additional cluster of about 20 cells, all with conspicuously high expression of *Il1rl1* (which encodes the IL-33 receptor chain IL-33Rα), was removed from the study because it was unrelated to the other clusters and instead

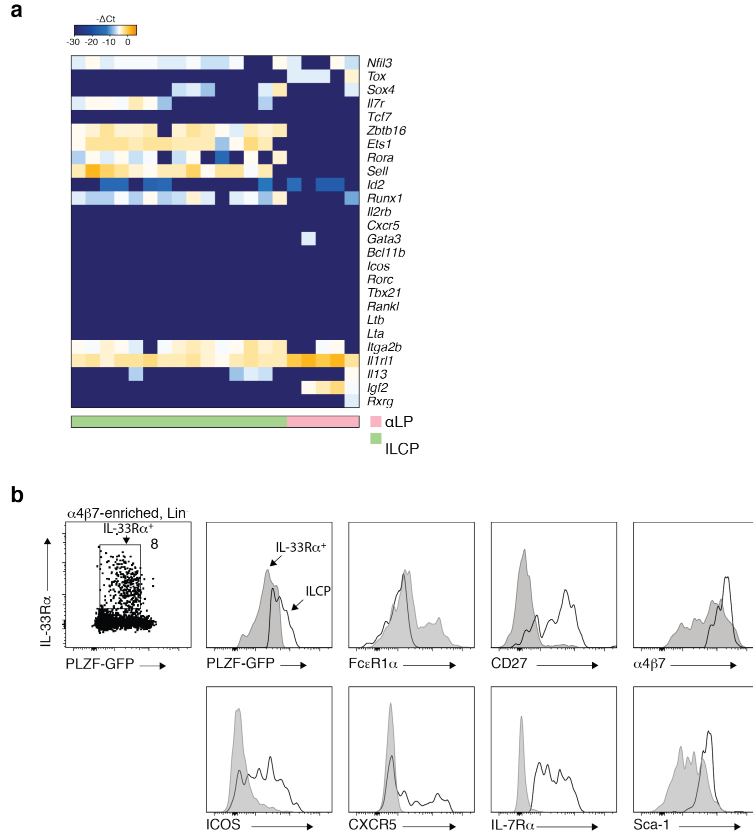

Figure 3.5: $\alpha 4\beta 7^{+}$IL-33Rα^{hi} cells represent contaminating mast cell precursors. (**a**) Biomark analysis of the *Il33r*$^{+}$ cluster excluded from the study suggested that they were not innate lymphoid cells, as evidenced by the nearly uniform lack of *Tcf7* and *Tox*, as well as other key markers. (**b**) FACS analysis suggested the mast cell nature of these cells. $\alpha 4\beta 7$-MACS-enriched fetal liver cells obtained from the *Zbtb16*-GFPCre reporter strain were gated as Lin^{-} cells (this is the same population that was used to sort single αLP and ILCP) and stained for IL-33Rα. The IL-33Rα^{+} cells expressed intermediate amounts of PLZF (GFP) and $\alpha 4\beta 7$, possibly explaining why they were found as contaminants in the Biomark analysis of 'purified' αLP and ILCP. 16% of these cells expressed the mast cell marker FcϵRIα, indicating that they were mast cell lineage precursors.

seemed to represent contaminating mast cell precursors with low expression of $\alpha 4\beta 7$ and PLZF (**Fig.3.5**). Thus, this analysis identified further heterogeneity among precursor cells and generated a 'blueprint' of their temporal sequence during ILC development.

3.1.3.3 Early developmental transitions

To facilitate analysis of the clusters identified above, we generated a condensed heat map of gene expression in all 299 single cells, limited to a set of 20 key informative genes. Consistent with LPs' being early precursors to ILCPs and LTiPs, there was sparse expression of genes encoding transcription factors and cytokines specific for these lineages in the A clusters. For example, *Zbtb16*, *Tcf7*, *Gata3*, *Bcl11b*, *Cxcr5*, *Rorc*, *Rora* and *Tbx21* were not expressed by clusters AI-AIII (**Fig.3.6**). In contrast, clusters AI-AIII expressed genes encoding transcription factors linked to early ILC and LTi development, including *Id2*, *Tox*, *Nfil3*, *Sox4*,

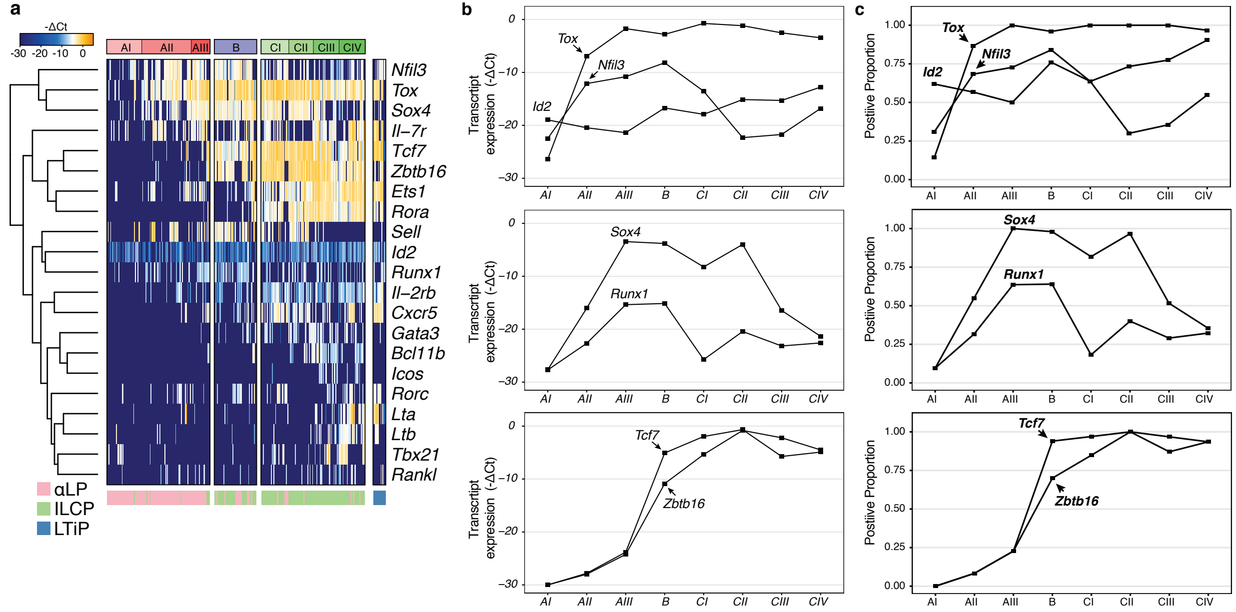

Figure 3.6: Clusters define the developmental progression of key transcription factors. (**a**) Single-cell multiplex quantitative PCR (as in Fig. 3.3) of transcripts from genes encoding products with known or anticipated roles in ILC development, ordered by hierarchical clustering of αLPs and ILCPs as in (Fig. 3.3), and of LTiPs (far right); below, sorted cell type (key, bottom left). (**b**) Transcript expression (average ΔCt values) for genes encoding products that define early developmental transitions in each cluster. (**c**) Expression frequencies of key transcription factors in αLP and ILCP transcription-state clusters define their developmental progression. In each cluster, the proportion of cells with detectable levels of transcript is calculated for genes defining early developmental transitions. Similar to the trends observed in average transcript levels by cluster, *Id2* is first expressed at a low level in AI, followed by *Tox* and *Nfil3* in AII, *Sox4* and *Runx1* in AIII, and finally by Tcf7 and Zbtb16 in B.

Ets1, *Runx1*, *Tcf7* and *Zbtb16* (**Fig.3.6a**). That conclusion was confirmed by plotting of the average mRNA expression per cell (**Fig.3.6b**) and the frequency of cells expressing genes encoding these transcription factors in each cluster (**Fig.3.6c**). Notably, a distinct temporal pattern of expression could be inferred from these plots. Thus, cells in cluster AI had low expression of *Id2*, but most lacked *Tox* and *Nfil3*. Nearly all cells in clusters AII and AIII, however, expressed *Tox* and *Nfil3*. Cells in clusters AI and AII lacked expression of *Ets1*, *Sox4* and *Runx1*, whereas a majority of cells in AIII expressed these genes. Finally, *Tcf7* and *Zbtb16* were not expressed by clusters AI-AIII (αLPs) but were widely expressed in cluster B and the C clusters (ILCPs) (**Fig.3.6**). We measured low *Id2* expression across clusters AI-AIII and found a tendency toward more frequent and higher expression in cluster

B and clusters CI-CIV (**Fig.3.6**), in line with the suggestion that increased *Id2* expression is correlated with commitment to innate lineages[59].

Thus, the Biomark analysis suggested that the temporal patterns of expression of these transcription factors were precisely regulated. Furthermore, unlike Tox, whose expression remained prevalent, *Nfil3* expression was ultimately reduced in ILCP clusters, consistent with its temporally limited requirement as suggested by late gene ablation experiments[84].

3.1.3.4 Bifurcation between ILC and LTi branches.

Cluster B comprised the most mixed representation of αLP and ILCP and seemed to be a developmental transition state linking early developmental events and specification to the ILC lineage versus the LTi cell lineage. Notably, cells in cluster B expressed *Tcf7* and *Zbtb16* but largely lacked expression of genes encoding ILC lineage-specific factors (**Fig.3.6**). In fact, the expression of *Tcf7* and *Zbtb16* was lower in cluster B than that in ILCP clusters (**Fig.3.6**), which confirmed the conclusion that cluster B corresponded to a transitional stage. A similar pattern was found for *Id2*, suggestive of developmental continuity between αLP clusters and ILCP clusters, with cluster B probably including the first cells to acquire *Zbtb16* expression before specification to the ILC lineage. Although the induction of *Tcf7* and *Zbtb16* seemed to be nearly synchronous, a fraction of cluster B cells expressed *Tcf7* without *Zbtb16* (**Fig.3.6**), which suggested that this fraction might have included cells destined to the LTi cell lineage. In fact, detailed analysis of cells in cluster B that expressed *Tcf7* but not *Zbtb16* showed that most of these cells had a tendency to express genes encoding factors associated with the LTi cell lineage, including *Rorc*, while conspicuously lacking expression of *Rora*, which is expressed in more mature LTiPs (**Fig.3.7**). In contrast, cells in cluster B that expressed both *Tcf7* and *Zbtb16* rarely expressed *Rorc* at that stage and instead displayed some expression of genes encoding factors associated with ILC lineages, such as

Gata3, while they lacked *Rora* expression (**Fig.3.7**); this suggested that they were less mature than most ILCPs. Thus, we concluded that cluster B represented the stage of bifurcation of the αLP into the LTi cell and ILC lineages.

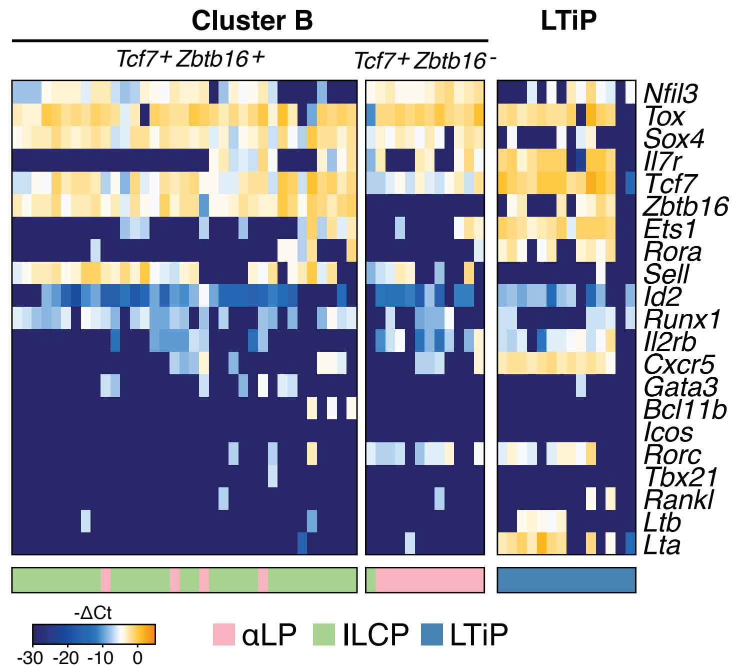

Figure 3.7: Transitional cluster B contains two distinct subsets on the basis of expression of *Tcf7* and *Zbtb16*. Transcript expression by cells from cluster B that express both *Tcf7* and *Zbtb16* ($Tcf7^{+}Zbtb16^{+}$) or express *Tcf7* but not *Zbtb16* ($Tcf7^{+}Zbtb16^{-}$) (Fig. 3.3), and of LTiPs (right).

3.1.3.5 ILC lineage differentiation originates in ILCP

Cells in the ILCP clusters (CI-IV) showed signs of ILC maturation and expressed genes encoding ILC lineage-defining transcription factors and cytokines (**Fig.3.6**). These cells had almost universally high expression of *Zbtb16*, *Tcf7* and *Id2*, and additional transcripts further distinguished them from the developmentally intermediate cluster B. Almost all cells in ILCP clusters expressed *Ets1* and *Rora* and tended to have low *Nfil3* expression. The ILCP clusters delineated by hierarchical clustering probably reflected sequential stages of ILC maturation, rather than association with particular ILC lineages. Cells in clusters CI and CII had high expression of *Sell* and *Sox4* that diminished in clusters CIII and CIV. Conversely, cells in clusters CIII and CIV had higher expression of *Ets1* and *Il7r* than that of cells in clusters CI and CII. Characteristically, cells in clusters CIII and CIV more frequently expressed genes encoding ILC lineage-defining transcription factors, including *Tbx21*, *Bcl11b* and *Rorc*, than did cells in clusters CI and CII. Our finding of the expression of genes encoding ILC lineage-defining transcription factors in multiple ILCP clusters indicated that we were probably capturing developmental stages of

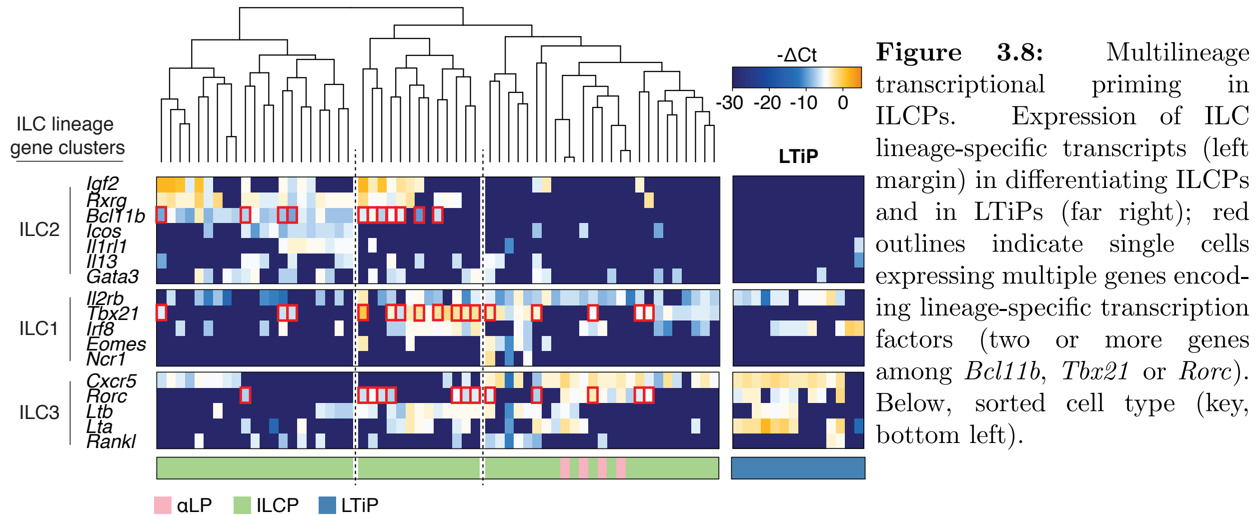

Figure 3.8: Multilineage transcriptional priming in ILCPs. Expression of ILC lineage-specific transcripts (left margin) in differentiating ILCPs and in LTiPs (far right); red outlines indicate single cells expressing multiple genes encoding lineage-specific transcription factors (two or more genes among *Bcl11b*, *Tbx21* or *Rorc*). Below, sorted cell type (key, bottom left).

ILC differentiation. Furthermore, cells that expressed genes encoding ILC lineage-defining transcription factors in later clusters (clusters CIII and CIV) tended to express genes encoding other lineage-associated cytokinesand transcription factors and thus seemed to be more differentiated.

3.1.3.6 ILCPs undergo multilineage transcriptional priming

To better assess the range of cells among the ILCP clusters that seemed to be differentiating toward ILC lineages, we compiled a subset of cells that expressed genes encoding ILC lineage-associated factors (**Fig.3.8**). Specifically, we identified cells with ILC1, ILC2 or ILC3 markers by their expression of *Tbx21* (ILC1) or *Bcl11b* (ILC2) or at least two genes among *Cxcr5*, *Rorc*, *Lta* and *Ltb* (ILC3). In this population, we observed many cells coexpressing genes encoding markers of different ILC lineages, such as *Tbx21* and *Bcl11b*, or *Tbx21* and *Cxcr5*, or *Tbx21* and *Rorc*, and even cells expressing multiple markers of the three different lineages. In total, among 120 total cells in ILCP clusters, we found 60 cells that expressed genes encoding lineage-differentiation markers, of which nearly one third (19) coexpressed genes encoding markers of different lineages.

The multilineage priming noted above was substantiated by the expression of genes encoding additional lineage-specific factors (**Fig.3.8**). In particular, the simultaneous expression of *Bcl11b* and *Icos* was associated with expression of canonical ILC2 genes, including *Il1rl1* and *Il13.* We also observed increased expression of *Gata3* in *Bcl11b*-expressing cells, although *Gata3* was expressed throughout ILCPs without strict lineage association (as shown before by flow cytometry[60]). Across cells in ILCP clusters, we observed a relatively strong correlation between the expression of *Rxrg* and *Igf2* and that of *Bcl11b.* The expression of *Tbx21* was associated with higher expression of *Il2rb* and, in some cases, with expression of *Eomes* and *Ncr1* (which encodes the activating receptor NKp46). We also found that *Irf8* expression seemed to be associated with *Tbx21* expression in differentiating ILCPs. Finally, we found that expression of *Rorc* was associated with high expression of *Cxcr5*, as well as with expression of *Lta*, *Ltb* and *Rankl.* Generally, expression of *Il2rb* and *Cxcr5* was observed throughout the ILCP clusters, with higher expression and greater frequency of expression associated with the expression of genes encoding other ILC1 and ILC3 factors. There was substantial overlap between the expression of genes encoding ILC1 and ILC3 factors, in particular for the expression of *Tbx21* and *Cxcr5*, *Lta*, *Ltb* and *Rankl.*

The results noted above were in contrast to the expression profile of LTiPs in the same fetal livers (**Fig.3.7 and 3.8**). These cells showed no evidence of multilineage priming. In particular, they conspicuously lacked expression of *Gata3*, *Bcl11b* or *Tbx21*, which further supported the proposal that they follow a distinct lineage-differentiation pathway.

Collectively, the expression of genes encoding multiple lineage factors in ILCP clusters was suggestive of multilineage potential. This further confirmed the proposal that cells navigating ILC lineage 'decisions' were in the ILCP population. Moreover, it suggested that during the initial lineage 'decisions', the relationships among distinct ILC lineage factors are more complicated than might have been anticipated from studies of helper T cells, for example.

3.1.3.7 Transcriptional programs correlate with lineage decisions

To functionally evaluate the relevance of the transcriptional programs noted above in ILCPs, we assessed their lineage potential in single-cell cultures. We sorted fetal ILCP subsets on the basis of their expression of the cytokine receptor chain CD122 (IL-2Rβ), ICOS and the chemokine receptor CXCR5 (**Fig.3.9a**) and studied their progeny in single-cell cultures. We found that these subsets showed some bias toward the corresponding ILC1 or ILC2 program (**Fig. 3.9b** (top row)). Thus, the earliest precursors with a bias toward the ILC1 lineage or ILC2 lineage could be discerned at the ILCP stage by their CD122^{+}CXCR5^{-} profile or ICOS^{hi} profile, repectively (**Fig.3.9a**). However, the biases were incomplete, indicative of

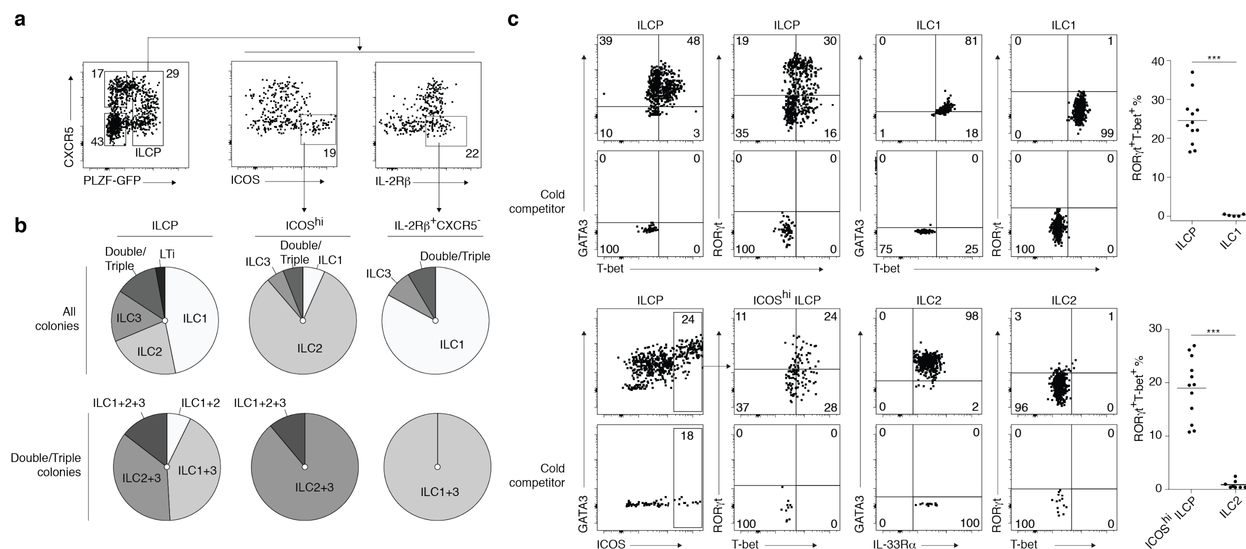

Figure 3.9: ILCP subsets with biased progeny in single-cell cultures. (**a**) Flow cytometry of fetal liver ILCPs, identified by GFP (PLZF) expression (as in Fig. 2) and sorted on the basis of expression of CXCR5, ICOS and IL-2Rβ. Numbers adjacent to outlined areas indicate percent cells in each subpopulation. (**b**) Distribution of single colonies and double or triple colonies (top) and the composition of double or triple colonies (bottom) from each progenitor cell. ILCP, n = 425 colonies; IL-2Rβ^{+}CXCR5 ILCP, n = 82 colonies; ICOS^{hi} ILCP, n = 150 colonies. (**c**) Intracellular staining of various transcription factors in fetal ILCPs at E14 (n = 12; gated as Lin^{-}IL-7R$\alpha^{+}\alpha4\beta7^{+}$ PLZF^{+}) and in ILC1 cells from mature adult liver (n = 6; gated as CD3ϵNK1.1^{+}CD49a^{+}) or ILC2 cells from adult bone marrow (n = 7; gated as Lin^{-}IL-7Rα^{+}Thy1^{+}Sca1^{+}), in the presence of unlabeled antibody (cold competitor) added before the conjugated antibody used for staining (bottom row of each group) or in the absence of such unlabeled antibody (top row of each group). Numbers adjacent to outlined areas or in quadrants indicate percent cells in each. Each symbol (far right) represents an individual mouse; small horizontal lines indicate the mean. *P $<$ 0.0001 (two-tailed Student's t-test). Data representative of at least three experiments (**a**), are pooled from one to six independent experiments (**b**) or are representative of two independent experiments (**c**).

the residual multipotency of these populations. For example, while ICOShi ILCPs generated mostly ILC2 colonies, they also gave rise to some single ILC1 and ILC3 colonies, as well as to dual and triple colonies (**Fig.3.9b**). In contrast, CD122$^+$CXCR5$^-$ ILCPs mostly generated mainly ILC1 colonies but also generated some single ILC3 colonies as well as dual colonies (**Fig.3.9b**). Even the ILCPs that produced dual colonies maintained a corresponding bias as, for example, all dual colonies originating from ICOS^{hi} precursors included an ILC2 colony, and those originating from a CD122$^+$CXCR5 precursor included an ILC1 colony (**Fig.3.9b, bottom row**). Together with the multilineage priming observed at the transcriptional level, these observations indicated that lineage polarization was proceeding by stepwise restriction of alternative programs from multipotential precursors, which ultimately led to the canonical polarized lineages. Using intracellular staining of transcription factors, we further confirmed that a substantial fraction of fetal ILCPs coexpressed T-bet, RORγt and GATA-3 or ICOS, indicative of multilineage priming, whereas mature ILCs were strictly limited to expression of their lineage-specific transcription factors (**Fig.3.9c**). This scenario is different from current models of the polarization of helper T cells, wherein acquisition of cytokine effector programs does not typically involve intermediates with mixed lineage patterns.

3.1.4 Discussion

Using the *Zbtb16*-GFP reporter system, we have identified the stage at which the αLP bifurcates into ILCP or LTiP during early fetal lymphopoiesis. We also used single-cell multiplex quantitative PCR analysis to produce a high-resolution map of the development of innate lymphocyte lineage. We emphasize that the proposed developmental progression discussed below is inferred on the basis of the continuity of gene expression between clusters but remains to be experimentally demonstrated.

We found that *Zbtb16* and *Tcf7* were simultaneously upregulated in cluster B at the bifurcation of the ILC and LTi cell lineages, with *Tcf7* expression marking both lineages

and *Zbtb16* expression identifying the ILC lineage. A fraction of the precursor cells that expressed *Tcf7* but did not express *Zbtb16* expressed *Rorc* and seemed closer to LTiPs than to ILCPs. Notably, cells in cluster B lacked expression of *Rora*, which is characteristically induced later in development, in further support of the conclusion that cells in cluster B navigate the bifurcation between the LTi cell lineage and ILC lineage.

The A clusters before the induction of *Zbtb16* and *Tcf7* must therefore represent earlier precursor cells, on the basis of the expression of *Id2*, *Tox*, *Nfil3*, *Runx1*[17,58,61,82,84,87,88,144], which has been linked to both lineages. Id2 seemed to be the earliest expressed transcription factor-encoding gene with substantial expression in cluster AI, a stage at which *Nfil3* and Tox transcripts were barely detected. Expression of *Nfil3* and *Tox* rapidly ascended during the transition to cluster AII and reached maximal levels in cluster AIII. These findings stand in apparent contrast to a published report showing that *Nfil3* can bind to and induce Id2 and that ablation of *Nfil3* can be complemented by Id2[84] but are consistent with the presence of *Id2* transcripts reported in arrested precursor cells lacking expression of *Nfil3* or *Tox*[84,88]. Nfil3 might therefore exert a positive feedback loop rather than a primary trigger for *Id2* expression. The expression of *Nfil3* and that of *Tox* also seemed to be induced nearly simultaneously. Thus, a report that Nfil3 can induce *Tox* and that ablation of *Nfil3* can be complemented by Tox[58] might also reflect a positive feedback mechanism.

Additional transcription factor-encoding genes that were induced early after *Id2*, *Tox* and *Nfil3* but before *Zbtb16* and *Tcf7* included *Sox4*, *Runx1* and *Ets1*. Although the function of the transcription factors encoded is currently unknown, Sox4 has been associated with the development of fetal $\gamma\delta$ T cells in conjunction with TCF-1[97], whereas Runx1 has been shown to be important for the development of NK cells and LTi cells[144], and Ets1 has been associated with NK cell development[107]. Thus, these factors are probably integral components of the early transcriptional network of innate lymphocytes.

Clusters CI-CIV defined several stages of ILCPs, with cluster CI associated with the

induction of *Rora*, which was maintained in all ILC lineages, although it seems to be required only for ILC2 cells[25,115]. Cluster CII was associated with *Gata3* expression, which was maintained at a high level during the remaining ILCP stages and ultimately is downregulated in mature ILC1 and ILC3 cells[60]. Therefore, transient but high *Gata3* expression is an intrinsic developmental event that might distinguish ILC3 cells from LTi cells. Clusters CIII and CIV were marked by emergence of the expression of genes encoding lineage-specific factors. Notably, there was a distinct and extensive pattern of coexpression of genes encoding factors from the three different lineages in nearly a third of these cells, which indicated that a phase of multilineage transcriptional priming preceded the polarization of mature lineages. In contrast, the LTiPs did not go through this singular process and directly expressed Rorc and other genes encoding attributes of the LTi cell lineage without coexpression of genes encoding alternative ILC lineage markers. The extent of multilineage priming revealed by our study goes beyond the coexpression of low levels of T-bet and RORγt by GATA3$^+$ ILC precursors detected in the fetal intestine by flow cytometry[66]. Indeed, we found that multilineage priming included an extended list of genes encoding canonical markers of all three lineages. ILCPs sorted on the basis of the expression of genes encoding some of these lineage differentiation markers showed a relative bias in differentiation toward the corresponding lineages after single-cell culture, although these subsets also maintained some multipotency, as shown by the generation of mixed colonies. These findings suggested that the terminal differentiation of ILCPs into polarized ILC lineages is more complex than previously envisaged. Instead of directly acquiring one of three programs, precursors of ILCs first activated multiple effector programs simultaneously and then progressively turned off priming of alternative lineages. In the absence of exogenous polarizing cytokines, such a developmental strategy can take advantage of the well-established antagonistic effects between these programs[99]. In ILCPs, external or internal inputs that might influence lineage 'decisions' include retinoids, which have been shown to favor type 3 innate lymphocytes[46,140], or Notch signals, which

are needed for ILC2 cells[18,96]. Multilineage transcriptional priming has been proposed as a more general strategy for developmental 'decisions', such as the differentiation of various hematopoietic lineages from a common progenitor[145–148]. It has not been widely reported for programs used in the differentiation of $CD4^+$ helper T cells into the T_H1, T_H2 or T_H17 subset of helper T cells, which instead rely on polarizing cytokines released during infection or allergy[99], although mixed transcriptional patterns have been observed in cells cultured under mixed cytokine conditions[149].

Our studies further emphasize that although they share many functional properties, ILC3 cells and LTi cells have distinct precursors and different developmental histories. Only precursors of ILC3 cells transited through a stage of high expression of *Zbtb16* and *Gata3* with multilineage priming at the single-cell level. Our results should encourage studies aimed at further elucidation of the different functions of LTi cells and ILC3 cells, as illustrated, for example, by a report that ILC3 cells and LTi cells have different topographical locations in the lamina propria[42].

In summary, our study has allowed the identification of several previously unknown developmental transitions and transcription factors sequentially associated with the lineage progression of innate lymphocytes. In particular, our results characterized in cellular and molecular detail the previously poorly defined bifurcation of lymphoid precursors into LTiPs and ILCPs. They have also demonstrated differential mechanisms of the acquisition of polarized effector programs by these lineages.

Author contributions

I.E.I, H.G., M.G.C. and A.B. designed the experiments; I.E.I. performed single cell sorting and culture experiments; S.C. designed and performed the lymphoid Biomark assay; H.G. performed computational analysis of the Biomark experiments; M.G.C. designed and performed experiments; A.D. supervised computational analysis; R.G. supervised Biomark experiments; A.B. supervised experiments; and I.E.I., H.G. and A.B. wrote the manuscript

with contributions from all authors.

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Competing Financial Interests

The authors declare no competing financial interests.

Accession codes. GEO: microarray data, GSE76407.

3.1.5 Materials and Methods

3.1.5.1 Mice

Zbtb16-GFP reporter mice were generated in our lab as previously described and contain an IRES after the last exon of *Zbtb16*, followed by an eGFP-Cre fusion gene[60]. For fetal ontogeny experiments, the morning a vaginal plug was identified was counted as embryonic day 0 (E0). Mice were housed in a specific pathogen free environment at the University of Chicago and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

3.1.5.2 Preparation of cell suspensions

Fetal livers were mechanically dissociated through a 70m cell strainer and resuspended in HBSS (Gibco) containing 0.25% BSA (Sigma-Aldrich) and 5mM sodium azide (Sigma-Aldrich).

3.1.5.3 Flow Cytometry

Cell suspensions were pre-incubated with BD Fc Block for 10 minutes on ice. Fetal liver cells were pre-enriched for $\alpha4\beta7$-expressing cells unless otherwise indicated. For pre-enrichment of $\alpha4\beta7^+$ cells, fetal liver or bone marrow cells were stained with allophycocyanin (APC)-conjugated antibody and subsequently bound to anti-APC microbeads (Miltenyi Biotec). The cells were then enriched using the autoMACS (Miltenyi Biotec), positive selection double sensitive program. Lineage (CD3ϵ, CD11c, CD19, NK1.1, TCRβ, Ter119 and GR-1) depletion of fetal liver or bone marrow cells was accomplished by incubation of cells with biotinylated Lineage antibodies and then bound to streptavidin conjugated microbeads (Miltenyi Biotec) and separated using the autoMACS depletion sensitive program. Fluorochrome or biotin conjugated monoclonal antibodies (clone in parentheses) against mouse

$\alpha4\beta7$ (DATK32), CCR6 (29-2L17), CD3ϵ (145-2C11), CD4 (GK1.5), CD8α (53-6.7), CD11c (N418), CD19 (6D5), CD27 (LG.3A10), CD45.1 (A20), CD45.2 (104), CD49a (Ha31/8), CD90.2/Thy1.2 (53-2.1), CD122 (5H4), CD127/IL-7Rα (A7R34), CXCR5(L138D7), FcϵRIα (MAR-1), GR-1 (RB6-8C5), ICOS (C398.4A), IL-33Rα/ST2 (DIH9), NK1.1 (PIK136), NKp46 (29A1.4), Sca-1 (D7), TCRβ (H57-597), Ter119 (TER-119), GATA3 (TWAJ), RORγt (Q31-378), T-bet (4B10), mouse IgG1/κ (MG1-45), mouse IgG2a/κ (MG2a-53), rat IgG2b/κ (RTK45-30) were purchased from Biolegend, eBioscience, or BD Biosciences unless otherwise noted. The D-9 antibody against PLZF was conjugated to Alexa-Fluor 647 using the labeling kit from Molecular Probes Life technologies. For intracellular staining, cells were fixed and permeabilized using the Foxp3 Transcription Factor Staining Buffer Set (eBioscience). Cells were then blocked with unlabeled isotype control before addition of fluorochrome-conjugated anti-transcription factor antibodies. As negative control, a 20-fold excess of unlabeled anti-transcription factor antibody was added prior to the conjugated antibody ("cold" competition). Data was acquired on a LSRII (BD Biosciences) or sorted using a FACS Aria II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

3.1.5.4 Single-cell cultures

Stocks of OP9 and OP9-DL1 stromal cells were a gift from J. C. Ziga-Pflcker. All experiments were performed in Opti-MEM with GlutaMAX (Gibco) containing 10% FCS (Gibco), 1% penicillin/streptomycin (Gibco), and 60mM 2-mercaptoethanol (Sigma-Aldrich) and maintained in a 37°C incubator (Thermo Scientific) with 5% CO2. Stromal cells were plated at 70% confluency. Before addition of lymphocytes, stromal cells were irradiated (1,500 rad) and culture media supplemented with murine IL-7 (25 ng ml^{-1}; R&D Systems) and SCF (25 ng ml^{-1}; BioLegend). Fetal liver lymphocytes were enriched for $\alpha4\beta7$ by MACS and single-cell sorted onto 96 well plates containing stromal cells and cytokines as described above. Cultures were analyzed after 6 or 10 days of culture and only colonies with more than 10

CD45.2$^+$ cells were considered.

3.1.5.5 Biomark

Cells were sorted in 96-well qPCR plates in 10μl of CellsDirectTM One-Step qRT-PCR Kit (Life Technologies), containing mixtures of diluted primers (0.05X final concentration, see Table 3.1 and 3.2). Preamplified cDNA was obtained after reverse transcription (15min at 40°C, 15s at 50°C and 15s at 60°C), and preamplification (22 cycles : 15min at 95°C, 4s at 60°C), and diluted 1 : 5 in TE pH8 Buffer (Ambion). Sample mix was as follows : diluted cDNA (2.9 μl), Sample Loading Reagent (0.29 μl, Fluidigm), TaqmanUniversal PCR Master Mix (3.3μl, Applied Biosystem) or Solaris qPCR Low ROX Master Mix (3.3 μl, GE Dharmacon). Assay mix was as follows: Assay Loading Reagent (2.5μl, Fluidigm), Taqman (2.5μl, Applied Biosystem) or Solaris (2.5μl, GE Dharmacon). 48.48 or 96.96 dynamic array integrated fluidic circuit (IFC, Fluidigm) was primed with control line fluid, and the chip was loaded with assays (either Taqman or Solaris) and samples using an HX IFC controller (Fluidigm). The experiments were run on a BiomarkHD (Fluidigm) for amplification and detection (2min at 50°C, 10min for Taqman reagents or 15min for Solaris reagents at 95°C, 40 cycles : 15s at 95°C, 60s at 60°C).

3.1.5.6 Analysis of single-cell multiplexed qPCR data

Independent single-cell qPCR experiments were performed for αLP, ILCP, and LTiP from two different pools of PLZF-GFPCre E15 fetal livers. Cells not expressing detectable levels of all three housekeeping genes Actb, Gapdh, and Hprt were removed from further downstream analysis. To appropriately compare measurements of distinct cells, expression levels of each gene for a given cell were normalized with respect to the average housekeeping gene expression level for that cell. Specifically the cycle threshold value for each gene, Ctg, was converted to the measure $-\Delta Ctg = <CtHKG> - Ctg$, the difference between the average housekeeping

gene cycle threshold and the gene cycle threshold. For quantification purposes, genes that were not detected were given a -ΔCt value of -30, close to the minimum value detected. Hierarchical clustering was performed using the Euclidian distance metric with complete-linkage agglomeration.

Visual inspection of the intercellular distances used for hierarchical clustering corroborates the strong distinction between αLP and ILCP clusters and the similarities between adjacent clusters in the prescribed developmental order (**Fig.3.4**). We performed permutation analysis to empirically evaluate the significance of our clustering assignments. Specifically, we calculated the sum of square Euclidean distances from the group mean for each cluster and used the total sum of squares within groups (SSW) value to compare our clustering assignment to 10,000 randomly permuted clustering assignments. We found the SSW of our clustering assignment to be substantially lower than those of all the random permutations, implying $P < 1e\text{-}4$. We further directly compared all pairs of clusters using the same approach and similarly found that all clusters were significantly distinguishable from one another with $P < 1e\text{-}4$. Finally, we performed hierarchical clustering with each gene iteratively removed from our complete dataset to evaluate the stability of our clustering method. We found that hierarchicalfig intercellular relationships were widely preserved under these perturbations. For instance, in 85% of all cell pairs, the number of branches connecting these pairs of cells to their common branch point on the clustering dendrogram were changed by less than 2 on average. Hierarchical clustering and analysis of qPCR data was performed with custom scripts using the base packages in R (v3.1.2) and heatmap displays were generated using the NMF package (v0.20.6).

3.1.5.7 Statistical analysis

Two-tailed Student's t-test was performed using Prism (GraphPad Software) *p<0.05; **p<0.001; ***p<0.0001.

Table 3.1: List of Solaris assyas used for the Biomark

Actb	AX-057827	*Hif1a*	AX-040638	*Rora*	AX-040430
Adam10	AX-041092	*Hprt1*	AX-045271	*Rorc*	AX-042209
Ahr	AX-044066	*Icos*	AX-044614	*Runx1*	AX-048982
Batf	AX-049093	*Ifngr1*	AX-043702	*Sox4*	AX-043251
Bcl11a	AX-062256	*Il12rb*	AX-046594	*Tbp*	AX-041188
Bcl11b	AX-055053	*Il13*	AX-046599	*Tbx21*	AX-040669
Ccr8	AX-042455	*Il17rb*	AX-049623	*Tcf7*	AX-062388
CD27	AX-057305	*Il18r1*	AX-046652	*Tcfe2a*	AX-062970
CD4	AX-049309	*Il1r1*	AX-046644	*Tet2*	AX-058965
Csf2	AX-043206	*Il1rl1*	AX-040418	*Tnfrsf11*	AX-042660
Cxcr5	AX-055590	*Il23r*	AX-053621	*Tnfrsf14*	AX-046033
Cxcr6	AX-047116	*Il2ra*	AX-041866	*Tnfrsf1a*	AX-060201
Egr1	AX-040286	*Il2rb*	AX-042082	*Tnfrsf1b*	AX-043973
Eomes	AX-063563	*Irf1*	AX-046743	*Tnfrsf9*	AX-043951
Ets1	AX-050997	*Lat*	AX-043878	*Tp53*	AX-040642
Gapdh	AX-040917	*Lck*	AX-048368	*Zbtb16*	AX-040219
Gata3	AX-040670	*Nfatc1*	AX-054700	*Zbtb7a*	AX-065288
Gfi1	AX-043458	*Nfil3*	AX-063246	*Tnfrsf1a*	AX-060201
Hes1	AX-060177	*Numb*	AX-046935		

Table 3.2: List of Taqman assyas used for the Biomark

Aes	Mm01148854_g1
Bcl2	Mm00477631_m1
c-myc	Mm00487804_m1
Cbfb	Mm01251026_g1
Cdkn1c	Mm01272135_g1
Dtx1	Mm00492297_m1
Ebf1	Mm01288946_m1
Fbxo27	Mm01179110_m1
Fgf15	Mm00433278_m1
Foxo1	Mm00490672_m1
Glis3	Mm00615386_m1
H19	Mm01156721_g1
Id2	Mm0011781_m1
Igf2	Mm00439564_m1
Igfbp4	Mm00494922_m1
Il7r	Mm00434295_m1
Irf8	Mm00492567_m1
Itga2b	Mm00439741_m1
Itga4	Mm00439770_m1
Lef1	Mm00550265_m1
Lmo4	Mm00495373_m1
Megf10	Mm01257625_m1

Ncr1	Mm01337324_g1
Notch1	Mm00435249_m1
Notch2	Mm00803069_m1
Pax5	Mm00435497_m1
Pira11	Mm04206919_gH
Ptprk	Mm00436070_m1
Rag2	Mm00501300_m1
Rank	Mm00437132_m1
Rbpj	Mm01217627_g1
Runx3	Mm00490666_m1
Rxrg	Mm00436411_m1
Sell	Mm00441291_m1
Spon2	Mm00513596_m1
Tle1	Mm00495643_m1
Tle2	Mm00498094_m1
Tle3	Mm00437097_m1
Tle4	Mm01195172_m1
Tnfsf1	Mm00440228_gH
Tox	Mm00455231_m1
Tsc22d3	Mm01306210_g1
Zbtb7b	Mm00784709_s1
Zbtb7c	Mm00628174_m1

3.2 Overexpression of PLZF

3.2.1 Summary

PLZF plays a seminal role in NKT development and its expression in T cells leads to the expression of an NKT effect program in naïve T cells. PLZF has also been shown to specifically mark an early ILC progenitor and is required for both ILC1 and ILC2. However it is not clear whether PLZF is sufficient to direct the ILC program in other lineages. Here we attempt to overexpress PLZF during early ILC development to assess its sufficiency in driving the ILC program.

3.2.2 Introduction

Promyelocytic leukemia zink finger (PLZF, encoded for by *Zbtb16*) was first identified in a patient with a rare form of acute promyelocytic leukemia, where PLZF was fused to *Rara*[150]. It is highly expressed in the brain, endocrine glands and male reproductive tissue[151]. Interestingly PLZF plays a crucial role in early development of limbs and axial skeletal patterning through the control of *HoxD* gene expression[152,153]and in the adult male in the maintenance of spermatogonia[154,155].

In the immune system PLZF is the master transcription factor of NKT cells[156,157]. Loss of *Zbtb16* results in a severe reduction of NKT in the thymus and the periphery. In NKTs PLZF is induced indirectly by TCR signaling via the upregulation of Egr1 and Egr2, which directly bind the promoter of *Zbtb16*[158]. On the other hand *Cd4*-promoter driven overexpression of PLZF resulted in the acquisition of an effector phenotype in CD4 single-positive T cells with low CD62L and high CD44 expression[156,159].

Our lab has shown that PLZF also is transiently expressed at the ILCP stage of ILC development and *Zbtb16*$^{-/-}$ mice showed defects in both ILC2 and ILC1[60], indicating its requirement for the development of both ILC lineages. However it is not clear whether

PLZF is also sufficient to direct the ILC program as has been shown for NKT. To address this question we tried two different methods of overexpressing PLZF.

Here we used a conditional retroviral and a transgenic overexpression system[160] to achieve high PLZF expression in ILC precursors. Our results failed to show significant diversion of lineages, although the level of expression achieved may have been limiting.

3.2.3 Results

3.2.3.1 Retroviral Overexpression of PLZF

Retroviral overexpression of genes offers a relatively quick way to express a gene of interest and assess its function. We used a conditional retroviral overexpression system, with which translation of a gene of interest is prevented until a fluorochome, in this case GFP, is excised by Cre recombinase (**Fig.3.10a**). In order to express PLZF during ILC development we transducing *Il7r*^{Cre}, since the IL-7R is upregulated at the CLP stage.

We succeeded in expressing PLZF promiscuously as expected. However we observed little change in NK populations and in T cell populations. This may mean that PLZF was not sufficient to induce ILC to induce the ILC fate, or that PLZF levels were not high enough as suggested by in *Cd4*-promoter driven PLZF transgenic mice. *Sell* (the gene encoding CD62L) is di-

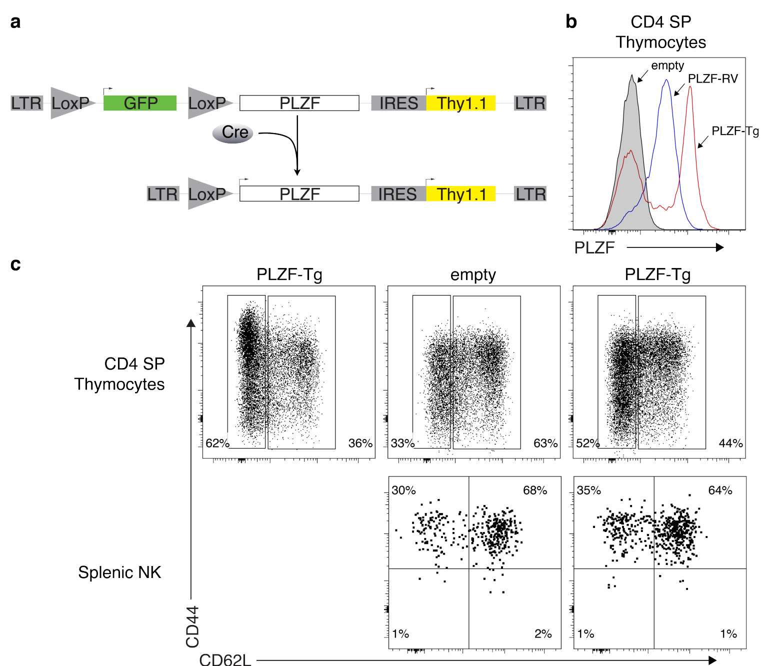

Figure 3.10: Retroviral overexpression of PLZF. (**a**) Schematic of the retroviral construct. (**b**) Expression levels of PLZF in CD4 single positive thymocytes. (**c**) CD44 and CD62L expression in CD4^{+} splenocytes and splenic cNK (CD3ϵ^{-}NK1.1^{+}).

rectly repressed by PLZF, we therefore also assessed CD62L expression in cells. The expression of CD62L was not reduced to the same amount in transduced CD4 single positive splenocytes as compared to *Cd4*-promoter driven transgenic CD4 single positive splenocytes (**Fig.3.10c**).

By overexpressing PLZF we hypothesized that PLZF can redirect other lineages to the ILC program. We therefore also looked at cNK cells in the spleen to see whether they exhibit an altered phenotype. However cNK did not appear to downregulate CD62L (**Fig.3.10c**).

While this result may suggest that PLZF is not sufficient for driving the ILC program, it is also possible that this approach did not induce high enough levels of PLZF.

3.2.3.2 TARGATT-PLZF Transgenic Mice

As an alternative to the retroviral overexpression system we created a PLZF transgenic mouse, by using the TARGATT system, where the phage integrase$_{\Phi}$C31 mediates recombination between attB and attP sites, allowing for the site specific targeting of a gene of interest[161]. Using this targeting system we integrated a chicken beta actin promoter-LoxP-Stop-PLZF cassette into the Rosa26 locus.

We crossed TARGATT$^{\text{PLZF}}$ mice to $Il7r^{Cre}$ mice to allow for the conditional expression of PLZF at the CLP stage, where the IL-7R is first upregulated. PLZF was faithfully expressed only in cells of the lymphoid lineage, but not in cells of the myeloid lineage, indicating successful targeting of the transgene expression (**Fig.3.11a**). However, similar to the retroviral overexpression system, in CD4$^+$ splenic T cells, PLZF expression in TARGATT$^{\text{PLZF/PLZF}}$ mice did not reach the same level as in *Cd4*-promoter driven PLZF transgenic mice. In the bone marrow precursors PLZF expression was less than half in αLP and ILC2P compared to ILCP (**Fig.3.11b-c**). We also did not observe any changes in the frequency of ILC precursor populations in the bone marrow.

We next asked whether PLZF expression was sufficient to redirect cNK cells to the

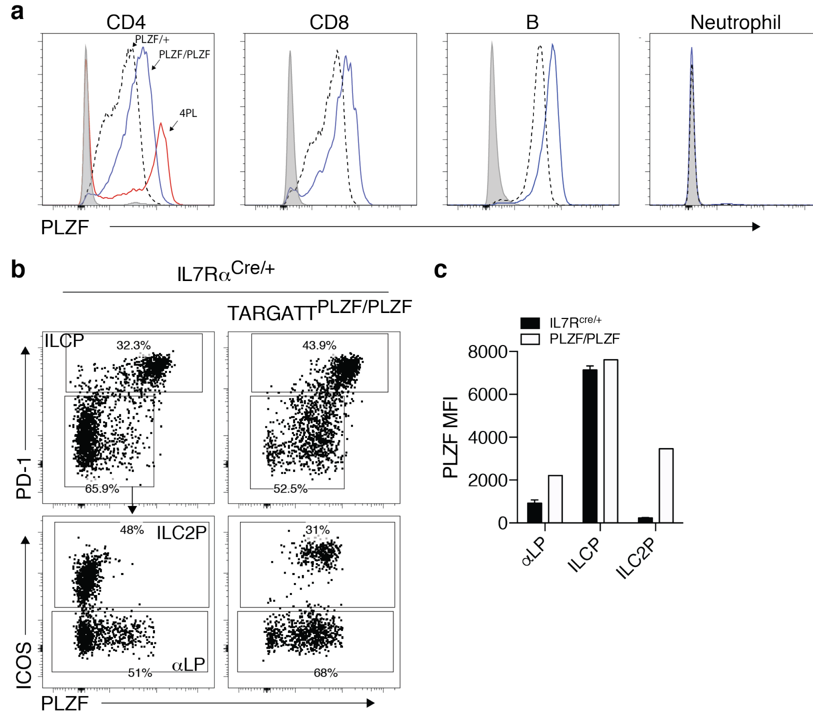

Figure 3.11: PLZF expression in TARGATT$^{PLZF/PLZF}$ mice does not reach levels of PLZF expression in ILCP. (**a**) PLZF expression in splenic CD4$^+$, CD8α^+ T cells, B cells and Neutrophils. *Cd4*-PLZF transgenic (PLZF-Tg), retroviral PLZF chimera (PLZF-RV)(**b-c**) PLZF expression in ILC precursor populations gated Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$ bone marrow.

ILC program. We saw no difference in the expression of CD62L or the total cell numbers of splenic cNK. We also asked whether liver cNK (CD3ϵ^-NKp46$^+$DX5$^+$) that ectopically express PLZF, would lose cNK specific marker expression and instead acquire ILC1 (CD3ϵ^-NKp46$^+$CD49a$^+$)specific markers. We did not observe differences in the frequency or total numbers of both ILC1 and cNK. The expression of the cNK specific marker Eomes and DX5 on Liver CD3ϵ^-NKp46$^+$ cells was not altered in PLZF transgenic mice.

3.2.4 Discussion

The promyelocytic leukemia zink finger plays a seminal role in NKT development, by promoting the development of effector programs of NKT1, NKT2 and NKT17[103,156]. This process occurs to a great extent through the repression of PLZF of Bach2[105], a transcriptional repressor of many hallmark T effector cytokines and transcription factors[106]. ILC and NKT lineage differentiation are reminiscent of each other and it is plausible that PLZF

acts similarly in ILC development to trigger the development of effector programs at the ILCP stage. We therefore asked whether PLZF is sufficient in ILC development to drive ILC effector programs.

We overexpressed PLZF in two different ways, using a conditional retroviral overexpression system and generating a conditional transgenic mouse. In both cases PLZF was successfully expressed in target tissues. The expression of CD44 and CD62L on ILCs or T cells was changed minimally. However the levels of PLZF induced in these cell types may have been insufficient. Therefore it remains possible that PLZF drives the ILC effector program.

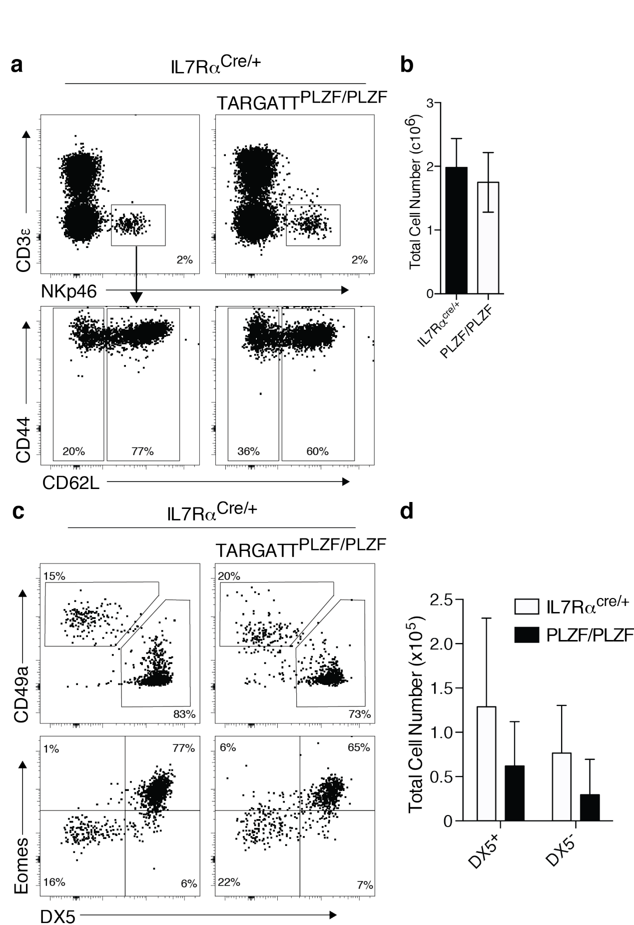

Figure 3.12: PLZF overexpression does not redirect Liver NK to the ILC1 program. (**a** CD62L and CD44 expression in splenic cNK cells) . (**b**) Absolute numbers of splenic cNK. (**c**) CD49a, DX5 and Eomes expression in Liver $CD3\epsilon^{-}NKp46^{+}$ cells. (**d**) Absolute numbers of Liver $DX5^{+}$ and $DX5^{-}$ Liver $CD3\epsilon^{-}NKp46^{+}$ cells.

The lack of high expression levels of PLZF in the retrogenic approach most likely is due to the size constraints of the retroviral vector as well as the viral packaging particle. The size of PLZF renders both viral transduction and packaging of viral particles inefficient. The retrogenic approach could potentially be improved by using a stronger promoter than the chicken β-actin promoter.

Studies, where PLZF was overexpressed in transfected cell lines have also shown that transfected cells more frequently underwent apoptosis than untransfected or control transfected cells[162]. It is therefore likely that in our system high PLZF expression is only permissible at the ILCP stage and that

earlier progenitors expressing high levels of PLZF may undergo apoptosis and therefore $PLZF^{hi}$ cells cannot be found in the periphery. In summary, we were able to faithfully overexpressed PLZF by two independent methods in the lymphoid lineage, but expression levels of PLZF were not high enough to efficiently drive the effector ILC phenotype. It therefore remains to be determined whether PLZF in fact is sufficient to drive the ILC effector phenotype.

3.2.5 Materials and Methods

3.2.5.1 Mice

TARGATT^{PLZF} mice were generated by PCR amplification using cDNA from sorted NKT as previously described[105,156]. *Zbtb16* was then subcloned into pBT346.6 (Applied Stem Cell, Inc.) using The minicircle containing the CAG-LoxP-Stop-LoxP-PLZF cassette was then injected into TARGATT^{TM} B6 mice that have the TARGATT site in the Rosa26 locus (Applied Stem Cell, Inc.) as previously described[161]. Il7r^{Cre} mice were a gift from Hans-Reimer Rodewald[163].

3.2.5.2 Retroviral Overexpression

pMGfI4 was a gift from Robert Brink[160]. The vector was modified by our lab (Jeff Bunker) to have Thy1 expressed constitutively after the IRES instead of human CD4. We cloned *Zbtb16* by PCR amplification of cDNA from sorted NKT using the forward primer 5'TCGCGGC-CGCCACCGAATTCGCCACCATGGATCTGACAAAG3' and the reverse primer 5'TGATTC-GAATCATCACCGGTTCACACATAACACAGGTAGAGG3'. We then cloned *Zbtb16* into the multiple cloning site using EcoRI and AgeI (New England Biolabs).

Virus was produced by transfecting PLAT-E cells with pMGfIThy1-PLZF. Bone marrow donor mice were treated with 150mg kg^{-1} body weight with 5-Fluorouracil (APP Pharmaceuticals) 3 days prior to harvesting the bone marrow. The bone marrow was cultured for 48h at 37°C in X-Vivo10 (Lonza) supplemented with 15% FCS, 1% penicillin/streptomycin, 100 ng mL^{-1} murine SCF, 10 ng mL^{-1} murine IL-3, and 20 ng mL^{-1} human IL-6 (all from Biolegend). Viral particles were mixed with polybrene (EMD Millipore) at 4 g mL^{-1} and the cells were spinfected at 800xg, 30°C for 90min. The spinfected cells were cultured for an additional 24h with cytokine and then analyzed and for transduction efficiency. Cells were mixed at a 1:1 ratio with freshly isolated bone marrow from Rag$^{-/-}\gamma$c$^{-/-}$ mice for better

reconstitution efficiency and then transferred into irradiated, congenically marked hosts.

3.2.5.3 Cell Culture

PlatE a gift from Robert Brink[160]. Cells were cultured with 1μg ml^{-1} Puromycine and 10μg ml^{-1} Blasticidin S (Sigma Aldrich) in DMEM (ATCC). Puromycine and B S lasticidin were removed for transfection and viral harvest.

3.2.5.4 Flow Cytometry

For analysis tissues were harvested and cells were resuspended in HBSS (Gibco) containing 0.25% BSA (Sigma-Aldrich), 0.65 mg L^{-1} sodium azide (Sigma-Aldrich) and 100mg L^{-1} DNaseI (Roche).

Lamina Propria cells were isolated by shaking the tissue twice with 5mM EDTA in RPMI (HyClone) at 37°C for 15min to remove the epithelium. The lamina propria was digested by shaking the tissue twice in RPMI supplemented with 0.5mg mL^{-1} Collagenase A (Roche) and 0.17mg mL^{-1} DNaseI (Sigma Aldrich) at 37°C for 30min.

Lamina Propria and Liver suspensions were additionally treated by resuspending in 5 ml of 40% Percoll (Sigma-Aldrich), and then centrifuged at 800g for 10 min.

Lineage was defined as CD3ϵ^+CD8α^+CD19$^+$TCRβ^+ in the lamina propria and as B220$^+$CD3ϵ^+CD11b$^+$CD11c$^+$CD19$^+$GR-1$^+$NK1.1$^+$TCRβ^+Ter119$^+$ in the bone marrow.

Cell suspensions were pre-incubated with BD Fc Block for 10 minutes on ice. Bone Marrow cells were pre-enriched for $\alpha 4\beta 7$-expressing cells unless otherwise indicated. For pre-enrichment of $\alpha 4\beta 7^+$ cells, bone marrow cells were stained with allophycocyanin (APC)-conjugated antibody and subsequently bound to anti-APC microbeads (Miltenyi Biotec). The cells were then enriched using the autoMACS (Miltenyi Biotec), positive selection double sensitive program. Fluorochrome or biotin conjugated monoclonal antibodies (clone in parentheses) against mouse $\alpha 4\beta 7$ (DATK32), CD3ϵ (145-2C11), CD4 (GK1.5), CD8α (53-6.7),

CD11c (N418), CD19 (6D5),CD44 (CD44), CD45.2 (104), CD49a (Ha31/8), CD62L (MEL-14), CD90.2/Thy1.2 (53-2.1), CD127/IL-7Rα (A7R34), DX5 (DX5), Eomes (Dan11mag), GR-1 (RB6-8C5), ICOS (C398.4A), NK1.1 (PIK136), NKp46 (29A1.4), PD-1 (29F.1A12), PLZF (R17-809), TCRβ (H57-597), Ter119 (TER-119), T-bet (4B10), mouse IgG1/κ (MG1-45) were purchased from Biolegend, eBioscience, or BD Biosciences unless otherwise noted. For intracellular staining, cells were fixed and permeabilized using the Foxp3 Transcription Factor Staining Buffer Set (eBioscience). Cells were then blocked with unlabeled isotype control before addition of fluorochrome-conjugated anti-transcription factor antibodies. As negative control, a 10-fold excess of unlabeled anti-transcription factor antibody was added prior to the conjugated antibody (”cold” competition). Data was acquired on a LSRII (BD Biosciences) or sorted using a FACS Aria II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

3.3 Tox2 and its Role in ILC Development and Function

3.3.1 Summary

The ILCP represents the earliest stage of ILC lineage trifurcation, but how this trifurcation is achieved is only partially understood. Here we investigate the role of Tox2, an HMG-box transcription factor that we found to be highly expressed at the ILCP stage, for its potential role in ILC lineage development. We find that Tox2 has modest repressive effects on ILC3s. In $Tox2^{-/-}$ mice we found an increase in ILC3 numbers and their potential to produce cytokines, but these differences in the ILC3 population did not have an observable impact on an in vivo model of infection.

3.3.2 Introduction

Tox2 is a member of a small family of high-mobility-group (HMG) box containing proteins, consisting of Tox2 itself, Tox, Tox3 and Tox4[164]. Tox was the first factor identified and named Thymocyte selection-associated HMG bOX (Tox) protein, due to its high expression in $CD4^+CD8^+$ double positive (DP) thymocytes. In T cells Tox enforces the CD4 lineage program through the regulation of ThPOK expression[165,166]. Interestingly Tox has also been shown to play a major role in ILC development. $Tox^{-/-}$ mice have defects in all ILC lineages, including cNK and LTi and has been suggested to act upstream of Id2[87,88].

Tox3 is highly expressed in the nervous system. In studies on neuronal cell lines Tox3 was shown to regulate the expression of neuronal survival factors in concert with the transcriptional regulator CITED1[167]. Mutations in Tox3 have also been observed in GWAS studies of breast cancer patients[168]. In in vitro studies Tox4 has been shown to associate with LEDGF, a reader of histone methylation, as well as PNUTS and HMGB1, which are components of the DNA damage response[169,170].

Tox2 was shown early on to be expressed in ovaries, although the function of Tox2 at this

site remains unclear[171]. A recent study focusing on human cNK cells, showed that Tox2 plays an important role in cNK development from umbilical chord blood precursors[90]. The study showed that *TOX2* knockdown in human cNK cells led to a downregulation of T-bet expression, by direct binding to the *TBX21* promoter, a major ILC lineage-defining transcription factor.

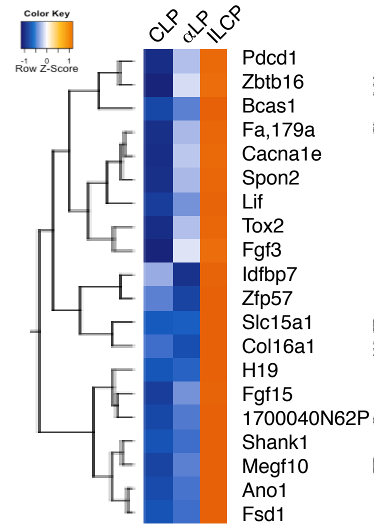

Figure 3.13: *Tox2* is highly expressed in ILCP. Cells itself is a heterogenouswere sorted from adult bone marrow for RNA sequencing. CLP (Lin$^-$IL-7Rα^+c-kit$^+$Sca1^{int}Flt3$^+$), αLP(Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$PLZF-GFP$^-$), ILCP (Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$PLZF-GFP$^+$).

We found that *Tox2* was highly expressed in ILCP, but not other ILC precursors, implicating that *Tox2* might act at the stage of ILC1, 2 and 3 lineage trifurcation. In this study we show that *Tox2* is specifically expressed in ILC precursors with a propensity to give rise to ILC3 and in ILC3 in the periphery. We also show that *Tox2* deletion has mild effects on ILC3 numbers and function, but that these changes have no significant effect in an in vivo infection model of Citrobacter rodentium.

3.3.3 Results

3.3.3.1 Tox2 is expressed in ILCP and peripheral ILC3

We have previously shown that the ILCP in itself is a heterogenous population, containing ILC lineage-primed precursors. Fractions of ILCP can be associated with the expression of lineage specific gene clusters. We therefore aimed to identify additional markers that act in ILCP to drive ILC lineage development. We performed RNA sequencing (RNAseq) on CLP (Lin$^-$IL-7Rα^+c-kit$^+$Sca1^{int}Flt3$^+$), αLP(Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$PLZF-GFP$^-$), ILCP (Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$PLZF-GFP$^+$) to identify additional transcription factors or makers that act in ILC development. Among the factors highly upregulated in ILCP only, we identified *Tox2* (**Fig.3.13a**).

We verified that *Tox2* is expressed exclusively in ILCP by performing qPCR on sorted precursors (**Fig.3.14a**). Interestingly we also saw that Tox2 was predominantly expressed in ILC1/ILC3 primed ILCP sorted from the fetal liver (**Fig.3.14b**), indicating that Tox2 may play a role in ILC1 or ILC3 lineage differentation, but not in ILC2. Analysis of peripheral lamina propria ILC popluations confirmed that Tox2 was expclusively expressed in type 3 ILCs, ILC3 and LTi, but not in other lineages (**Fig.3.14c**).

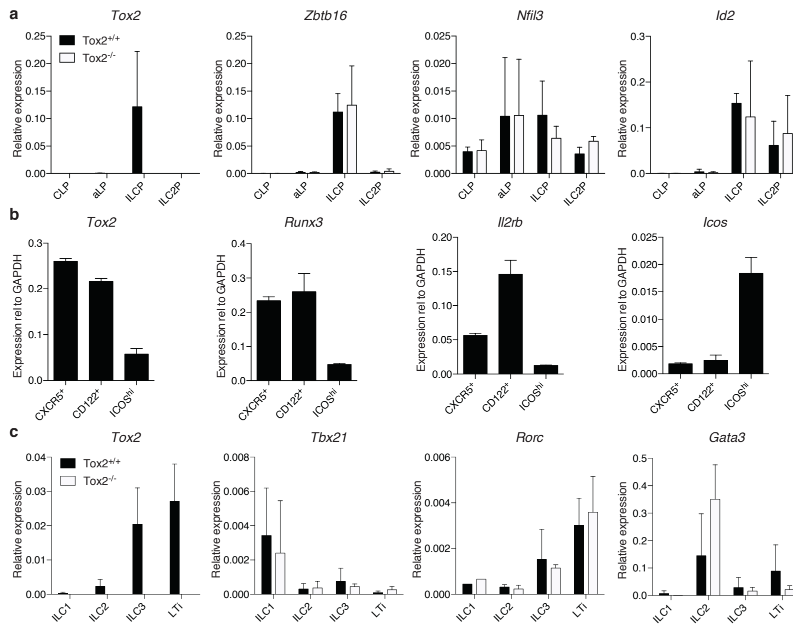

Figure 3.14: *Tox2* transcript is specifically expressed in ILC3. (**a**) qPCR on sorted bone marrow precursors. αLP and ILCP were sorted as in Fig.3.13. CLP were sorted Lin$^-$IL-7Rα^+Flt3$^+\alpha 4\beta 7^-$and ILC2P Lin$^-$IL-7R$\alpha^+\alpha 4\beta 7^+$PLZF-GFP$^-$ICOS$^+$. (**b**) Fetal Liver ILCP subsets. Fetal Liver ILCP (Lin$^-$IL-7R$\alpha^+\alpha 4\beta 7^+$PLZF-GFP$^+$) were further subsetted into CXCR5$^+$ (CXCR5$^+$ICOS$^-$), CD122$^+$ (CXCR5$^-$CD122$^+$) and ICOS^{hi} (CXCR5$^-$ICOS^{hi}). (**c**) qPCR on lamina propria ILC populations. ILCs were sorted (Lin$^-$Thy1$^+$IL-7Rα^+). To subset we used additional markers to distinguish ILC1 (KLRG-1$^-$NK1.1$^+$NKp46$^+$), ILC2 (KLRG-1$^+$NK1.1$^-$), LTi (KLRG-1$^-$NK1.1$^-$CCR6$^+$) and ILC3 (KLRG-1$^-$NK1.1$^-$CCR6$^-$). Data is compiled from three independent experiments.

3.3.3.2 Generation of a *Tox2*-knockout mouse

In order to study the function of Tox2 in ILC development we generated *Tox2*-knockout mice using the CRISPR/Cas9 system. *Tox2* is a complex gene with five different protein-coding tanscripts. We determined from the RNAseq we performed in CLP, αLP and ILCP that transcript *Tox2-002* was the predominant isoform expressed in ILCP (**Fig.3.15a**). Transcript *Tox2-004* could be detected in ILCP at lower levels. None of the transcripts share the same first exon and translation start site. It was therefore impossible to introduce a

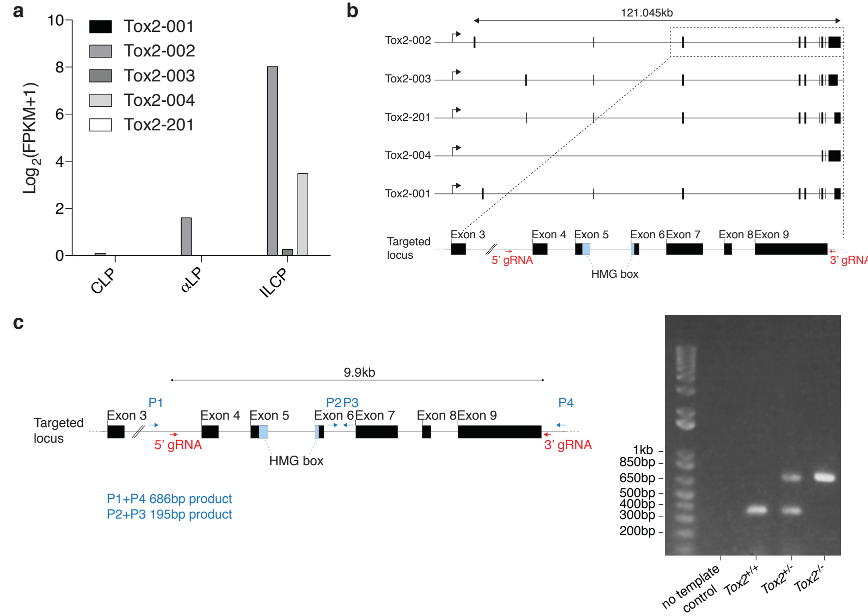

Figure 3.15: *Tox2* transcript is specifically expressed in ILC3. (**a**) Transcript expression in ILC precursors. (**b**) *Tox2* gene structure and guide RNA position. (**c**) PCR strategy for the detection of the deletion of exons 4-9.

deleterious deletion in the first exon. Also due to the relative size of the genetic region that encompasses the first three exons we opted instead to delete a region of 9.9kb encompassing exons 4-9. Exons 5 and 6 share the homeobox domain of Tox2, deletion of which would therefore prevent DNA binding of Tox2 (**Fig.3.15b**). We were able to generate the desired deletion as confirmed by PCR (**Fig.3.15c**) and sequencing (data not shown).

By qPCR of bone marrow precursor and peripheral ILC populations we also confirmed that we could not detect any Tox2 transcript in *Tox2*$^{-/-}$ mice (**Fig.3.14a**). By comparison with *Tox2*$^{+/+}$ populations we could not detect any significant changes in key transcription factor expressions. In the bone marrow we did not observe changes in the expression of key ILC developmental factors *Zbtb16*, *Id2* and *Nfil3* (**Fig.3.14a**). In the periphery we did not detect any changes in key lineage defining factors in ILCs. *Gata3*, *Tbx21* and *Rorc* leves were not significantly altered between *Tox2*$^{+/+}$ or *Tox2*$^{-/-}$ ILCs (**Fig.3.14c**).

3.3.3.3 Tox2 has modest effects on ILC3 frequency and function

Since *Tox2* is highly expressed in ILCP and in peripheral ILC3 and LTi, we asked whether deletion of *Tox2* has any effect on peripheral ILCs and specifically on type 3 ILCs. We did not notice any significant changes in peripheral ILC populations. The $\mathrm{Lin^{-}Thy1^{+}ROR\gamma t^{-}NKp46^{+}}$ population that contains both ILC1 and cNK and ILC2 ($\mathrm{Lin^{-}Thy1^{+}ROR\gamma t^{-}NKp46^{-}Gata3^{+}}$) were unaltered in *Tox2*$^{-/-}$ mice (**Fig.3.16a**). Among $\mathrm{Lin^{-}Thy1^{+}ROR\gamma t^{+}}$ ILC3 we further differentiated $\mathrm{NCR^{+}}$, $\mathrm{NCR^{-}}$ and LTi by CCR6 and NKp46 expression. We did not observe any significant differences in the frequency or absolute numbers among LTi or ILC3 subsets in *Tox2*$^{-/-}$ mice (**Fig.3.16a-b**). We also observed no significant functional changes in

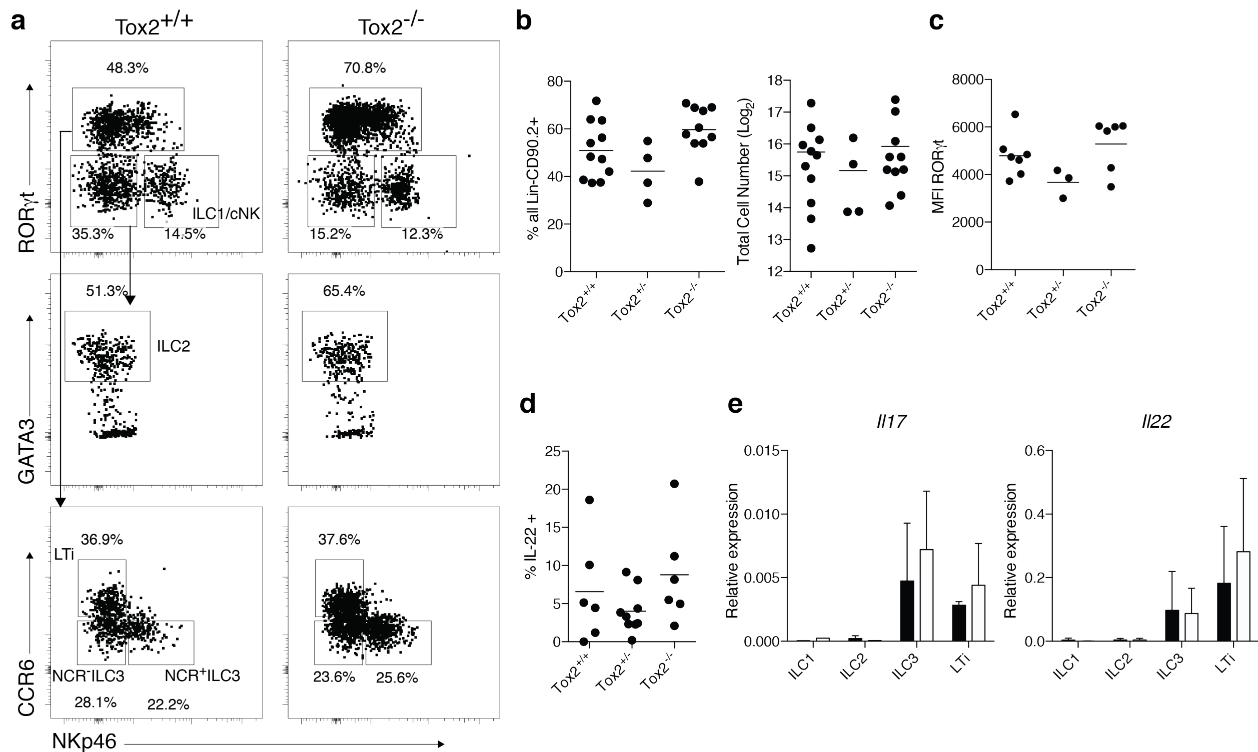

Figure 3.16: Tox2 does not have any effects on peripheral ILC populations and function in straight *Tox2*$^{-/-}$ mice. (**a**) Representative plots showing LP populations of ILCs form *Tox2*$^{+/+}$ or straight *Tox2*$^{-/-}$ mice. (**b**) ILC3 ($\mathrm{Lin^{-}Thy1^{+}ROR\gamma t^{+}}$) % of all ILC ($\mathrm{Lin^{-}Thy1^{+}}$) and absolute ILC3 cell numbers. (**c**) RORγt MFI among $\mathrm{Lin^{-}Thy1^{+}IL\text{-}7R\alpha^{+}KLRG\text{-}1^{-}NK1.1^{-}}$ ILC3. (**d**) IL-22 expression in LP ($\mathrm{Lin^{-}Thy1^{+}}$) cells. (**e**) qPCR for *Il22* and *Il17* on sorted LP ILC populations (see Fig.12). Data is compiled from at least three independent experiments.

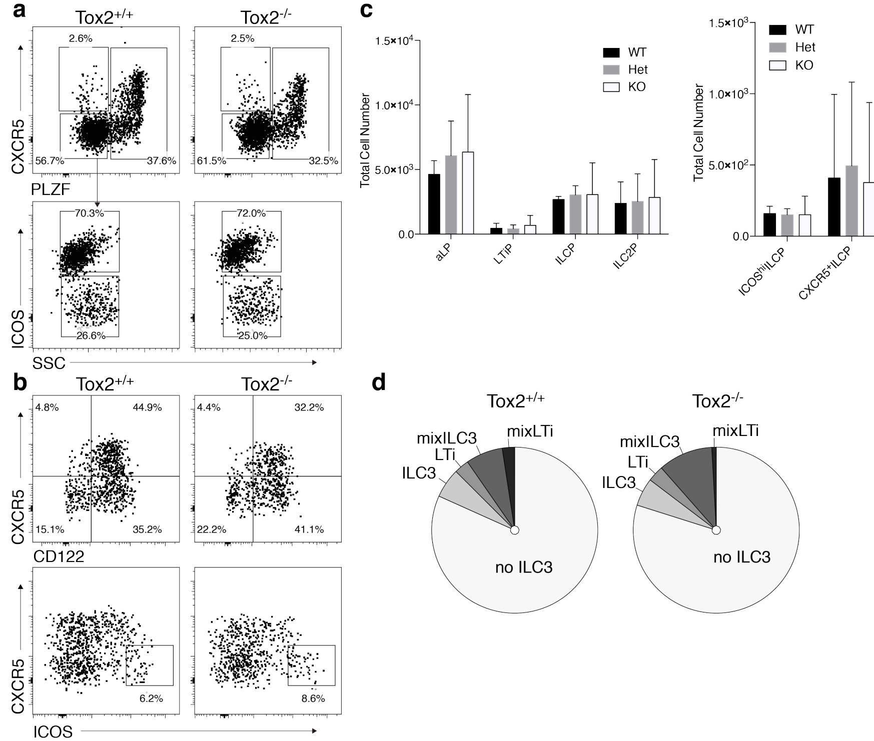

Figure 3.17: *Tox2*$^{-/-}$ ILC precursors are unaltered. (**a**) Bone Marrow ILC precursor populations. αLP (Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$CXCR5$^-$PLZF$^-$ICOS$^-$), LTiP (Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$CXCR5$^+$PLZF$^-$), ILCP (Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$PLZF$^+$), ILC2P (Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$CXCR5$^-$PLZF$^-$ICOS$^+$). (**b**) ILCP (gated as in (a)) subsets by CXCR5, ICOS and CD122 expression. (**c**) Total cell numbers of ILC precursors and ILCP subsets. (**d**) Single-cell culture of ILCP sorted from *Tox2*$^{+/+}$ and *Tox2*$^{-/-}$ fetal livers. Data was compiled from at least three independent experiments. For *Tox2*$^{+/+}$ 210 colonies grew with a cloning efficiency of 42.17% and for *Tox2*$^{-/-}$ 359 colonies grew with a cloning efficiency of 52.56%.

Tox2$^{-/-}$ ILC3, when we stained for IL-22 in total lamina propria ILCs. We also observed no changes in the expression of *Il22* and *Il17* by qPCR in LTi or ILC3.

In human studies it has been shown that Tox2 has an impact on cNK cell development from human chord blood precursors. In knockdown studies of *Tox2* it was found that Tox2 was required for T-bet expression. We found that this was not the case in murine *Tox2*$^{-/-}$ mice. Neither ILC1 nor cNK in the liver were altered in frequency or number and T-bet protein and message were the same in both *Tox2*$^{+/+}$ and *Tox2*$^{-/-}$ (**Fig.3.18**).

Since *Tox2* is highly expressed in ILCP we also asked whether deletion of *Tox2* has any effects on ILC precursor populations in the bone marrow. αLP, ILCP and ILC2P were not altered in frequency or numbers in *Tox2*$^{-/-}$ mice compared to *Tox2*$^{+/+}$ litter mates

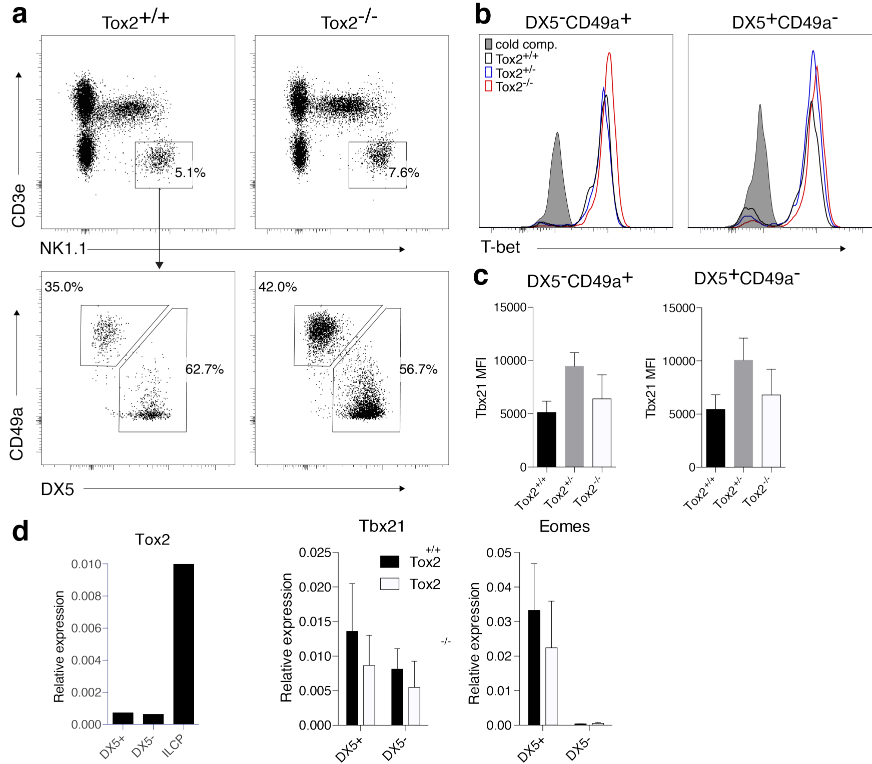

Figure 3.18: *Tox2*$^{-/-}$ cNK and ILC1 have normal T-bet expression. (**a**) Liver cNK were stained for CD49a and DX5. (**b-c**) T-bet protein expression and MFI among CD3ϵ^-NK1.1$^+$ cNK (DX5$^+$CD49a$^-$) and ILC1 (DX5$^-$CD49a$^+$). (**d**) qPCR of Tox2, T-bet and Eomes on sorted Liver cNK and ILC1.

(**Fig.3.17a, c**). We also noted no differences in lineage primed ILCP. CXCR5$^+$ILCP, which expressed *Tox2* at higher levels compared to ICOS^{hi} ILCP, were not altered in frequency or numbers (**Fig.3.17b-c**).

In order to assess whether *Tox2*$^{-/-}$ ILCP had an altered developmental potential we sorted ILCP from *Tox2*$^{+/+}$ or *Tox2*$^{-/-}$ fetuses into single-cell culture. After 6 days in culture we harvested the cells and analyzed them by flow cytometry for the expression of ILC-lineage specific markers as described previously[60]. We did not detect any differences in the propensity of either *Tox2*$^{+/+}$ or *Tox2*$^{-/-}$ ILCP to give rise to type 3 ILCs. Thus ILC precursors in *Tox2*$^{-/-}$ mice were not altered phenotypically or functionally.

3.3.3.4 Tox2 negatively regulates ILC3 numbers and function

We next asked whether *Tox2*$^{-/-}$ cells would have a competitive advantage or disadvantage over *Tox2*$^{+/+}$ cells in mixed bone marrow chimeras. We mixed *Tox2*$^{-/-}$ bone marrow at a 1:1 ratio with CD45.1 congenically marked *Tox2*$^{+/+}$ bone marrow and transferred the cells retro-orbitally into CD45.1/CD45.2 congenic hosts. 5 Weeks after bone marrow transfer we analyzed the chimeras for differences in ILC populations and cytokine expression. we noted that *Tox2*$^{-/-}$ bone marrow gave rise to a higher frequency of ILC3 compared to *Tox2*$^{+/+}$ bone marrow. Among total ILCs we also noted a higher frequency of ILC3 that produced IL-22 in *Tox2*$^{-/-}$ bone marrow, although not all chimeras exhibited this phenotype. In conclusion we detected mild effects of Tox2 on ILC3 numbers and function.

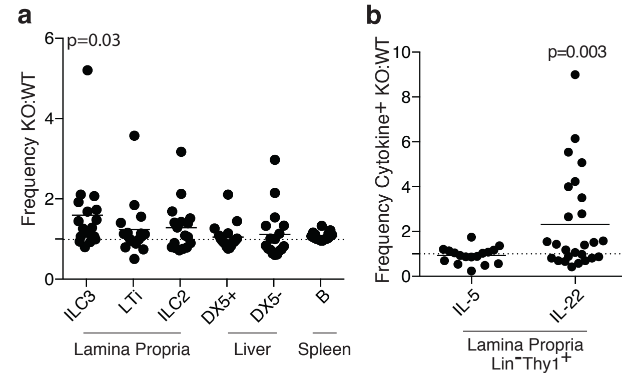

Figure 3.19: *Tox2*$^{-/-}$ ILC3 show moderate expansion and increase in IL-22 production in mixed bone marrow chimeras. (**a**) . (**b**) IL-22 staining of Lin^{-}Thy1^{+} lamina propria ILCs. Data is compiled from at least three independent experiments with 17 chimeras (a) and 26 chimeras (b).

3.3.3.5 *Tox2*$^{-/-}$ mice do not show altered clearance of C.rodentium infection.

ILC3s have been shown to play a seminal role the clearance of Citrobacter rodentium, a mouse-restricted pathogen that can be used as a model to study enterophathogenic or enterohemorhaging bacterial infections in vivo[172,173]. Especially at early time points during the infection, ILC3s provide large amounts of IL-22[29] which is required for the regeneration of the gut epithelium and production of antimicrobial peptides necessary for the clearance of C.rodentium[2].

We asked whether, due to increased ILC3 numbers and elevated IL-22 production, *Tox2*$^{-/-}$ mice would be protected in a C.rodentium infection model. We infected *Tox2*$^{+/+}$ and *Tox2*$^{-/-}$ mice with 2x10^{9}CFU per mouse after starving the animals overnight. We collected the weight of the mice as well as feces to assess fecal CFU at days 1, 3, 5 and 8 at which point the mice were sacked an analyzed for changes in ILC populations.

We found that *Tox2*$^{-/-}$ mice showed no significant differences in either weight loss or fecal CFU during the course of the experiment (**Fig.3.20a-b**). We did observe a trend towards reduced fecal CFU at day 3 post infection, but this difference was not statistically significant. We also did not observe an expansion of type 3 ILCs or changes in their capability to produce IL-22 after 8 days of C.rodentium infection (**Fig.3.20c-d**).

Overall we conclude that *Tox2* deficiency did not have any observable effect on the ability of ILC3 to contribute to the clearance of C.rodentium in vivo.

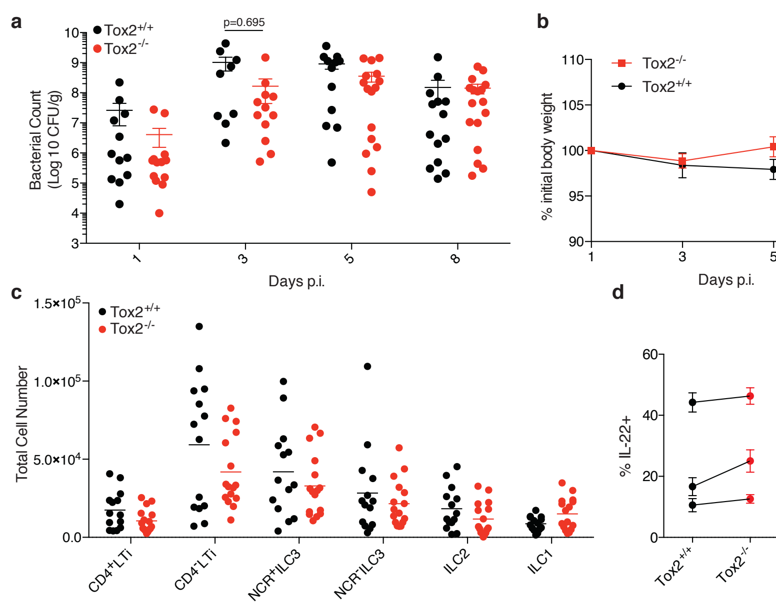

Figure 3.20: *Tox2*$^{-/-}$ mice are not protected from C. rodentium infection. Mice were infected with 2x10^{9} CFU after overnight starvation. (**a**) Fecal CFU/g of feces post infection. (**b**) weight loss post infection. (**c**) Absolute cell numbers of ILC populations in the lamina propria on day 8 post infection. (**d**) IL-22 production among Lin^{-}Thy1^{+}RORγt^{+} ILC3 in the lamina propria on day 8 post infection. The data is combined from 3 independent experiments with 13 *Tox2*$^{+/+}$ and 19 *Tox2*$^{-/-}$ mice total. Statistical significance was assessed using two-way Anova.

3.3.4 Discussion

Tox2 is a family member of Tox, which has been shown to play an essential role in ILC development. So far transcription factors such as *Bcl11b* and *Runx3* have been shown to

influence the development of ILC subsets in particular and both are expressed starting at the ILCP stage.

We have found that Tox2 is exclusively and highly expressed only at the ILCP stage in early ILC precursors, suggesting its role in the lineage trifurcation of ILCs. In fact Tox2 was expressed in ILC3 lineage-primed ILCP, but not in $ICOS^{hi}$ ILCP, which predominantly give rise to ILC2 and peripheral ILC3, suggesting that Tox2 plays a role in ILC3 development.

We successfully generated *Tox2* knockout mice and found that, although the effects are modest, Tox2 appears to repress the ILC3 program, leading to increases in ILC3s and ILC3 function in the absence of *Tox2* in competitive bone marrow chimeras. It is interesting that Tox2, as a negative regulator of ILC3s, is expressed in the same population, as opposed to other ILC lineages. One intriguing possibility is that Tox2 promotes the conversion of ILC3 to NCR^{+}ILC3 by repressing aspects of the ILC3 program. Tox2 however does not appear to suppress the major ILC3 defining transcription factor RORγt or upregulate the ILC1 defining transcription factor T-bet, which is required for the conversion to NCR^{+}ILC3, indicating that Tox2 has no major influence on the stability of the lineages.

We also showed that *Tox2* deficient ILC3 show an increase in IL-22 production, suggesting that it plays a role in setting the activation threshold of ILC3s. ILC3 have been shown to produce IL-22 at steady state, even in the absence of stimulation[63] and its dysregulation can lead to inflammation and tumor formation[174], indicating the necessity for a regulated expression pattern of IL-22.

A possible explanation for the weak phenotype of *Tox2*$^{-/-}$ mice could lie in the fact that other Tox2 family members may compensate for the loss of *Tox2*. Tox is also expressed at the ILCP stage, although its expression pattern is not restricted to the ILCP, like Tox2. Its broader function in ILC development also makes it unlikely that Tox can specifically compensate for the function of Tox2 in ILCs. The expression of Tox3 and 4 does not seem to be increased in αLP or ILCP over CLP in *Tox2*$^{+/+}$ cells by RNA sequencing. This

however does not exclude the possibility that either Tox3 or Tox4 could be upregulated in the absence of Tox2. Therefore it remains to be determined whether other Tox family members are upregulated in the absence of Tox2 expression and thus compensate for loss of *Tox2*.

In conclusion we have shown that Tox2 has modest effects on ILC3 population size and function and that it acts as a regulator of the ILC3 program, although it is likely that it acts together with other factors that have the capacity to compensate for its absence.

3.3.5 Materials and Methods

3.3.5.1 Mice

Tox2 knockout mice were generated using the CRISPR/Cas9 system as described previously[175]. The plasmid for Cas9 and guide RNA expression was obtained from Addgene. We used the guide RNA 5'GGCAGGTGTGGCATTTGTCGAGG3' located 5' to Exon4 and 5'GGCGTTGGGAGCGGGTAATCAGG3' located 3' to Exon9 to delete a 9.9 kb genomic region containing Exons 4-9 of *Tox2*. Guide RNAs were designed using the online open access tool CRISPRDesign (crispr.mit.edu).

Guide RNAs were ordered as DNA primers, annealed by boiling and slow cooling and ligated into pT7-gRNA (Addgene plasmid # 46759)in a one step reaction, containing BglII, SalI and BsmBI, NEB buffer 3, T4 ligase and T4 ligase buffer (all from New England BioLabs Inc.). The vector was linearized using BamHI (New England BioLabs Inc.) and guide RNAs were in vitro transcribed using the MEGAshortscript T7 kit (Invitrogen). guide RNAs were purified using the MEGAclear kit (Ambion). pT3TS-nCas9n (Addgene plasmid # 46757) was linearized by digesting with NotI (New England BioLabs Inc.) Cas9 mRNA was generated with mMESSAGE mMACHINE SP6 kit (Invitrogen)[175]. Cas9 RNA and guide RNAs were injected at 150ng μL^{-1} (Cas9) and 25ng μL^{-1} (each guide RNA). To detect the deletion we used the the primers P1 5'TTCATTGTTGCACAGCACAC3', P2 5'CCCTGCCTCTCTCTGTTCAC3', P3 5'CACTAAGGGGCAGGGTGTAA3', P4 5'CACTAAGGGGCAGGGTGTAA3' as shown in Fig.3.15c. Mice were back-crossed for at least 5 generations.

PLZF^{GFPCre} reporter[60] and C57BL/6 (Jackson Laboratory) mice were used as well.

3.3.5.2 RNA Sequencing

5-10x10^3 cells of CLP (Lin$^-$IL-7Rα^+c-kit$^+$Sca1^{int}Flt3$^+$), αLP(Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$PLZF-GFP$^-$), ILCP (Lin$^-$IL-7R$\alpha^+\alpha4\beta7^+$PLZF-GFP$^+$) were sorted from PLZF^{GFPCre} reporter mice (ILCP and αLP) or C57BL/6 mice (CLP). RNA was prepared using the Arcturus PicoPure RNA Isolation kit (Applied Biosystems) and reverse transcribed using the USB First-Strand cDNA Synthesis kit (Affymetrix). RNA was analyzed using the Illumina HiSeq system.

3.3.5.3 qPCR

Cells were sorted as indicated into TLC buffer (Quiagen) with 1% RNAse free 2-Mercaptoethanol (Sigma) at a concentration of 100cells μL^{-1}. RNA was isolated using cDNA was generated with the Superscript IV (Invitrogen). qPCR was performed using the TaqMan Assay (Thermo Fisher Scientific) (Table 3.3).

3.3.5.4 Bone Marrow Chimeras

Bone marrow chimeras were generated by mixing *Tox2*$^{-/-}$ and *Tox2*$^{+/+}$ CD45.1 congenically marked bone marrow at a 1:1 ratio. Cells were retro-orbitally injected into lethally irradiated (1000rad) mice. Mice were analyzed 5 weeks after reconstitution.

3.3.5.5 Single-Cell Cultures

Single-cell cultures were performed as previously described[60]. Cells were sorted as single cells into OptiMEM-Glutamax (Gibco) supplemented with 10%FCS, 1%Penicillin-Stremptomycine, 25ng mL^{-1} IL-7 and mSCF. Cells were cultured for 6 days and then harvested and analyzed by flow cytometry.

3.3.5.6 Flow Cytometry

For analysis tissues were harvested and cells were resuspended in HBSS (Gibco) containing 0.25% BSA (Sigma-Aldrich), 0.65 mg L^{-1} sodium azide (Sigma-Aldrich) and 100mg L^{-1} DNaseI (Roche).

Lamina Propria cells were isolated by shaking the tissue twice with 5mM EDTA in RPMI (HyClone) at 37°C for 15min to remove the epithelium. The lamina propria was digested by shaking the tissue twice in RPMI supplemented with 0.5mg mL^{-1} Collagenase A (Roche) and 0.17mg mL^{-1} DNaseI (Sigma Aldrich) at 37°C for 30min. Lamina Propria and Liver suspensions were additionally treated by resuspending in 5 ml of 40% Percoll (Sigma-Aldrich), and then centrifuged at 800g for 10 min.
Cell suspensions were pre-incubated with BD Fc Block for 10 minutes on ice. Bone Marrow cells were pre-enriched for $\alpha 4\beta 7$-expressing cells unless otherwise indicated. For pre-enrichment of $\alpha 4\beta 7^+$ cells, bone marrow cells were stained with allophycocyanin (APC)-conjugated antibody and subsequently bound to anti-APC microbeads (Miltenyi Biotec). The cells were then enriched using the autoMACS (Miltenyi Biotec), positive selection double sensitive program. Lineage was defined as $CD3\epsilon^+CD8\alpha^+CD19^+TCR\beta^+$ in the lamina propria, $B220^+CD3\epsilon^+CD11b^+CD11c^+CD19^+GR\ \text{-}1^+NK1.1^+TCR\beta^+Ter119^+$ in the bone marrow and $CD3\epsilon^+CD11c^+CD19^+GR\ \text{-}1^+NK1.1^+TCR\beta^+Ter119^+$ in the fetal liver.

Fluorochrome or biotin conjugated monoclonal antibodies (clone in parentheses) against mouse $\alpha 4\beta 7$ (DATK32), CCR6 (29-2L17), $CD3\epsilon$ (145-2C11), CD4 (GK1.5), $CD8\alpha$ (53-6.7), CD11b (), CD11c (N418), CD19 (6D5), CD45.2 (104), CD49a (Ha31/8), CD90.2/Thy1.2 (53-2.1), CD122 (5H4), CD127/IL-7Rα (A7R34), CXCR5(L138D7), DX5 (DX5), GR-1 (RB6-8C5), ICOS (C398.4A), NK1.1 (PIK136), NKp46 (29A1.4), PD-1 (29F.1A12), PLZF (R17-809), TCRβ (H57-597), Ter119 (TER-119), GATA3 (TWAJ), RORγt (Q31-378), T-bet (4B10), mouse IgG1/κ (MG1-45) were purchased from Biolegend, eBioscience, or BD Biosciences unless otherwise noted. For intracellular staining, cells were fixed and per-

meabilized using the Foxp3 Transcription Factor Staining Buffer Set (eBioscience). Cells were then blocked with unlabeled isotype control before addition of fluorochrome-conjugated anti-transcription factor antibodies. As negative control, a 10-fold excess of unlabeled anti-transcription factor antibody was added prior to the conjugated antibody ("cold" competition). Data was acquired on a LSRII (BD Biosciences) or sorted using a FACS Aria II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

3.3.5.7 Citrobacter Rodentium Infection

C. rodentium strain DBS100 (ATCC 51459; American Type Culture Collection) was grown in LB broth. Mice were starved overnight and orally gavaged with $2x10^9$ CFU of C.rodentium.

3.3.5.8 Statistical analysis

Two-way Anova was performed using Prism (GraphPad Software).

Table 3.3: List of Taqman assyas used qPCR on Tox2 *Tox2*$^{-/-}$ or *Tox2*$^{+/+}$ cells

Tox2	Mm01241014_m1
Zbtb16	Mm001176868_m1
Nfil3	Mm00600292_s1
Id2	Mm00711781_m1
Runx3	Mm00490666_m1
Il2rb	Mm00434268_m1

ICOS	Mm00497600_m1
Tbx21	Mm00450960_m1
Rorc	Mm01261022_m1
Gata3	Mm00484683_m1
Il22	Mm01226722_g1
Il17a	Mm00439618_m1

CHAPTER 4

DISCUSSION & FUTURE DIRECTIONS

4.1 Discussion

4.1.1 A Blueprint of ILC Development

In this study we established a blueprint of ILC development on the basis of the continuous expression of transcriptional profiles in single-cell ILC precursors. The clear definition of ILC precursors using the *Zbtb16*-GFP reporter strain allowed us to sort defined ILC precursor populations and perform single-cell multiplex qPCR. Through the analysis of more than 100 transcripts we establish a precise map of ILC developmental progression based on the continuity of the expression of key regulatory factors. We confirmed that Nfil3, Id2 and Tox are the earliest regulators of ILC development that are expressed in early clusters of αLP and that PLZF and Tcf-1 defined a transitional stage that marks the split of the ILCP and LTiP. We furthermore showed that the ILCP is the stage of lineage-trifurcation, a process which occurs through multi-lineage priming.

4.1.1.1 A Transitional Cluster of LTiP- and ILCP-Primed Precursors

Although individual precursor populations to LTi and ILCP were described previously, it was not clear what their last common precursor is and what specific characteristics might govern the bifurcation of ILCP and LTiP. We show here in single-cell cultures, that a late stage of the αLP, the Flt3$^{-}\alpha$LP, is the last precursor, that gives rise to both LTi and ILCs. The identification of the Flt3$^{-}\alpha$LP as the stage of ILC and LTi bifurcation, allowed us to identify a transitional cluster (cluster B) of mixed αLP and ILCP, that showed characteristics of both LTiP and ILCP. This transitional cluster was marked by the upregulation of *Zbtb16* and *Tcf7*. This cluster lacked expression of many ILC lineage-specific markers, such as *Rorc*, *Gata3*,

Bcl11b, *ICOS*, *Il2rb*, *CXCR5*, but showed expression of early ILC developmental factors, *Nfil3*, *Tox* and *Id2*, placing the cluster before the ILCP and LTiP. Cluster B could be divided into two major groups by the expression of *Zbtb16*. Interestingly *Zbtb16* negative cluster B cells showed almost uniform expression of *Rorc*, but were *CXCR5* and *Rora* negative, both markers that were expressed in LTiP, suggesting that the *Zbtb16* negative fraction is poised to differentiate into LTiP. *Zbtb16* expressing cluster B cells were uniformly *Gata3* low. *Sell*, which is negatively regulated by PLZF[103], was still expressed in *Zbtb16*$^+$ cluster B cells, indicating that this fraction of cells are an early ILCP.

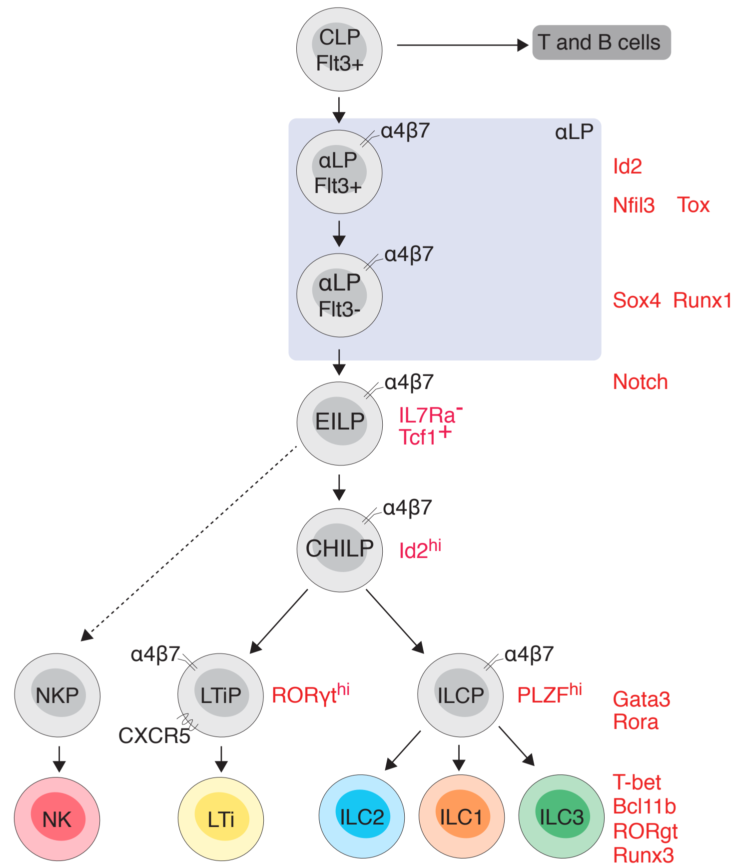

Figure 4.1: A Revised Model for ILC development. In this model the placement of the EILP is based on the expression of *Tcf7* and its capability to produce ILCs, LTi as well as cNK in transfers. However further proof is required for the placement of the EILP in this hierarchy. The exact characteristics and existence of the CHILP as a distinct precursor from the ILCP, LTiP and αLP also still needs to be determined, but most likely the CHILP will be a precursor that is RORγt$^-$PLZF$^-$ and expresses Tcf-1 and high levels of Id2, unlike the αLP.

These data shows that LTi and ILC3 indeed have distinct developmental pathways that can be differentiated based on *Zbtb16* expression. However, how *Zbtb16* is regulated in ILC development, is not clear. Furthermore *Tcf7* upregulation marked the transition to LTiP and ILCP, placing its expression at the late αLP stage, after the expression of *Nfil3*, *Tox* and *Id2*. Since *Tcf7* is also expressed in the EILP, it appears likely that the EILP is the stage directly preceding cluster B and is discussed below (see page 89).

Interestingly cluster B also expressed high levels of *Sox4* and *Runx1*, two transcription factors which heretofore have not been well described in ILC development. The expression of *Sox4* and *Runx1* at the transition to LTiP and ILCP, begs the question, whether both transcription factors are involved in the regulation of *Zbtb16*, *Rorc* and *Tcf7* expression and thus function as drivers of ILC and LTi lineage bifurcation.

4.1.1.2 *Id2*, *Nfil3* and *Tox*, Mark the Earliest Stages of ILC Development

Identification of cluster B showed that Runx1, Sox4 as well as Tcf-1 and PLZF belong to a group of transcription factors that act at the bifurcation of LTi and ILCs. The absence of *Tcf7*, *Zbtb16*, as well as ILC lineage-specific transcription factor expression marked a cluster of cells, that did express early ILC developmental factors, *Nfil3*, *Tox* and *Id2* (cluster A). In comparison to cluster B, cluster A therefore represents a developmentally earlier precursor population that almost exclusively consists of αLP. *Id2* is the earliest expressed transcription factor and is required for the repression of E-proteins, which drive the differentiation of both the B and T cell lineage[67]. The expression of *Id2* is rapidly followed by the induction of *Nfil3* and *Tox*.

This finding stands in contrast to findings, suggesting that early ILC developmental factor *Id2* is regulated by Tox and Nfil3 and thus is expressed as the consequence of the action of both transcription factors. In *Tox*-knockout CD4^{lo}SP thymocytes and LTi, *Id2* expression is reduced and retroviral transduction of Lin^{-}Ska-1^{+}cKit^{+} bone marrow with

Id2 resulted in the partial recovery of ILC precursors in *Tox* knockout bone marrow[87,165], claiming that Id2 functions downstream of Tox and that Tox can regulate *Id2* expression. However it is not clear whether Tox can directly bind the *Id2* promoter, and likely other factors are involved in its regulation.

Similarly *Nfil3*-deficient ILC precursors in the bone marrow or fetal liver show a marked reduction in *Id2* expression and Nfil3 can directly bind to the *Id2* promoter in ChIP of CHILP as well as peripheral LTi, which show high Nfil3 expression[84,113]. Overexpression of *Id2* in *Nfil3*$^{-/-}$ bone marrow rescued the *Nfil3*$^{-/-}$ phenotype. These data suggest that Tox and Nfil3 function in the regulation of *Id2* expression. However, since *Id2* is expressed before both *Tox* and *Nfil3*, it is more likely that both Tox and Nfil3 function in a positive feedback loop to enhance *Id2* expression. How *Id2* is induced during ILC development remains unknown and requires the investigation of the epigenetic landscape of *Id2*.

Possible regulators of *Id2* in T cells or B cells include Egr1 or activation of the Smad pathway, by TGF-β and the TGF-β family member, bone morphogenic protein 4 (BMP4)[176,177]. A predominant site for TGF-β production is in the gut, but TGF-β1 is also expressed in the bone marrow by stromal cells, where it could potentially act on CLP to upregulate Id2 and thereby initiate ILC development. In fact treatment of B lymphocyte precursors with TGF-β resulted in the upregulation of *Id2* and *Id3* as well as growth inhibition of these cells[178].

Tox expression has been suggested to be regulated by Nfil3 in CLP. *Nfil3*$^{-/-}$ CLP showed a significant reduction in *Tox* expression compared to *Nfil3*$^{+/+}$ CLP[58] and the *Nfil3*$^{-/-}$ phenotype could be rescued by *Tox* overexpression, suggesting that Nfil3 acts as a regulator of *Tox* expression. Whether Nfil3 in fact can directly bind the *Tox* promoter and regulate its expression is somewhat controversial as ChIP of Nfil3 in CD4^{-}LTi and murine lymphoblast cell line EL4 yield opposing results[58,84]. It therefore remains to be determined whether Nfil3 directly regulates *Tox* expression in early ILC progenitors at the

αLP stage, when *Tox* is first upregulated or whether there are other factors that regulate *Tox* expression[58,84].

How *Nfil3* is regulated during ILC development remains unclear. Studies in cell cultures of ILC precursors as well as peripheral ILC2 and 3 have shown that IL-7 can induce *Nfil3* expression[84] and in cNK cells *Nfil3* appears to be induced by IL-15 signaling[80], suggesting that perhaps common gamma chain signaling induces *Nfil3*.

In summary, we show that Nfil3, Tox and Id2 are members of an early ILC developmental network and have been shown, to some extent, cross regulate each other. However it remains to be determined how *Id2*, *Tox* and *Nfil3* are induced and regulated during ILC development. The precise definition of ILC precursors we have established facilitates the analysis of the promoter landscapes of sequential ILC precursors and can shed light on potential inter-genic interactions as well as the gene expression at different ILC developmental stages.

4.1.1.3 The ILCP Stage Is Marked by Multilineage Transcriptional Priming

PLZF expression, which marks the ILCP stage, is transient and lasts about four days, before PLZF is downregulated and differentiated ILC1s, ILC2s, and ILC3s emerge[60]. This relatively short period is characterized by a series of remarkable and rapid changes that lead to the priming of differentiated ILC lineages[55] (**Fig. 4.1**).

About half the ILCPs begin to display signs of priming toward ILC1, ILC2, and ILC3 lineages. Notably, many of them exhibit mixed lineage priming, whereby a single cell can co-express markers of two or three lineages as detected in multiplex transcriptomic experiments. For example, many cells coexpress *Gata3*, *Tbx21*, and *Rorc*, as well as other markers of differentiated lineages, such as *Bcl11b* and *Icos* (ILC2), *Lta/Ltb* and *Cxcr5* (ILC3), and *Il2rb* (ILC1) in mixed patterns[55]. This mixed pattern precedes the differentiated mature ILC lineages. Coexpression of intermediate levels of Gata3, T-bet, and RORγt has also been noted using FACS analysis of the ftILCP in the fetal intestine[66]. Notably, it is not observed in the

LTiP, which exhibits a pure type 3 cytokine program[55]. This multilineage transcriptional priming of ILCP stands in apparent contrast with the direct polarization of T_H1, T_H2, and T_H17 cells driven by exogenous cytokines and STAT signaling during immune responses[99], although mixed patterns have been observed under mixed cytokine culture conditions[149]. Multilineage patterns have also been reported in other hematopoietic precursors, suggesting that it is a common mechanism involved in multilineage decisions[145–148], whereby antagonistic lineage-specific factors are stochastically coexpressed and compete for dominance, in a process modulated by environmental clues. The ILCP stage is characterized by the sequential induction of a panoply of transcription factors that have critical functions in ILC development and are described below.

Gata3 protein is expressed at intermediate to high levels in about 70% of ILCPs[60], a much greater frequency than expected if Gata3 expression were strictly correlated with the ILC2 fate. In fact, in mice where floxed *Gata3* alleles were deleted with *Vav-Cre*, or in irradiation chimeras reconstituted with *Gata3*$^{-/-}$ fetal liver cells, most, though not all, ILCs were missing[101,102,116]. Thus, ILC2s and some ILC1s (particularly those expressing IL-7Rα in the thymus) were severely decreased. Intestinal lamina propria ILC1s were also depleted in *Ncr1-Cre Gata3*$^{fl/fl}$ mice[59]. Surprisingly, even though Gata3 has not been detected in the LTiP, or later in LTi, *Vav-Cre Gata3*$^{fl/fl}$ mice lacked LTiPs in the fetal liver and consequently had impaired development of Peyers patches and lymph nodes, suggesting that *Gata3* is expressed at low levels that have escaped detection. In contrast, *Gata3* does not influence early cNK development. Deletion of floxed *Gata3* alleles in the periphery of mice bearing a *CreER*^{T2} deleter allele and treated with tamoxifen resulted in loss of ILC2s but not ILC3s, indicating that *Gata3* is also essential to the maintenance of peripheral ILC2s, although the mechanism is unclear[102].

Rora is induced in most ILCPs after expression of PLZF and is also found in the LTiP[55]. It is maintained in peripheral ILCs and peripheral LTis. However, deletion of Rora selectively

impairs the appearance of ILC2s in peripheral tissues, as well as the frequency of ILC2Ps in the bone marrow, by unknown mechanisms, apparently without impact on other ILC types[25,115].

Bcl11b is expressed at the late ILCP stage and correlates closely with coexpression of *Icos* among differentiating ILCPs, and with the emergence of ILC2s but not other ILC types, indicating that *Bcl11b* is an early and specific marker of ILC2 commitment at the ILCP stage, as demonstrated using a *Bcl11b* reporter system or by single-cell transcriptomic analysis[55,117]. Although deletion of *Bcl11b* is embryonic lethal, irradiation chimeras reconstituted with $Bcl11b^{-/-}$ fetal liver and $Bcl11b^{fl/fl}$ mice bearing a $CreER^{T2}$ allele and treated with tamoxifen have been generated[117–119]. A recent analysis of single-cell precursors in *Vav-Cre* $Bcl11b^{fl/fl}$ mice has shown that the block in ILC2 development in fact occurs as early as the ILCP stage[124].

Most studies have demonstrated that *Bcl11b* plays a critical role in the expression or maintenance of *Il1rl1* (encoding the inducible ST2 chain of the IL-33 receptor), *Gata3*, and *Rora* and in the suppression of *Sox4*, *Rorc*, *Il23r*, and *Il17*. Thus, deletion of *Bcl11b* in mature ILC2s tends to deviate their differentiation toward the ILC3 lineage. In fact, most of these effects seem indirect and, as detailed below, may be mediated by *Gfi1*[120], which is itself a direct target of Bcl11b. The signals leading to *Bcl11b* induction in ILCPs have not been elucidated, but there is a striking analogy with the *Notch-Tcf7-Gata3-Bcl11b* cascade identified in thymocyte development[179] (**Fig.4.2**).

Gfi1 is broadly expressed in hematopoietic lineages and has well-established roles in promoting T_H2 differentiation and suppressing T_H17 differentiation[180,181]. *Gfi1* is increased in peripheral ILC2s and regulates their expression of *Il1rl1* and *Gata3*[120]. Interestingly, Gfi1 directly inhibits genes of the T_H17 module such as *Sox4*, *Il1r1*, and *Il17a*, implying that *Gfi1* regulates the balance and the function of ILC2 and ILC3 lineages. Mice constitutively lacking *Gfi1* also exhibit a defect in generation of bone marrow ILC2s and mature peripheral ILC2s,

indicating that *Gfi1* is active in development as well as in the peripheral maintenance of the ILC2 lineage[120].

Runx3 driven by its distal promoter is abruptly expressed at the ILCP stage in about 50% of ILCPs and persists at high levels in ILC1s and at intermediate levels in ILC3s but is not observed in ILC2s. A *Vav-Cre*-mediated deletion of floxed *Runx3* alleles showed that *Runx3* is essential for the development of ILC1s and ILC3s and partially essential for development of LTis but not CLPs, αLPs, or ILCPs[111]. Specifically, *Runx3* was essential for *Rorc* induction in ILC3s and for the survival of ILC1s. Because Runx3 and Gata3 are known to bind and antagonize each other at the protein level[182], the relative expression of these two proteins in ILCPs might be a critical factor for ILC2 versus ILC1 or ILC3 decision (**Fig.4.2**). A fraction of ILCP also showed expression of *Rorc* as well as *Tbx21*, highlighting that these precursors most likely are ILC1 or ILC3 primed.

Similarly the expression of Gfi1 and Bcl11b in a fraction of ILCP suggests that these particular precursors are primed to develop into ILC2. The identification of ILCP that show ILC-lineage primed expression modules indicated that ILC lineage differentiation already occurred at the ILCP stage. ILC lineage-primed precursors could in fact be distinguished extracellularly by ICOS, CXCR5 and CD122 (IL-2Rβ) staining[55]. ICOS^{hi}CXCR5^{-}CD122^{-} ILCP, which also express *Gata3* and *Bcl11b*, predominantly give rise to ILC2 in single-cell cultures, similarly CD122^{+}CXCR5^{-}ICOS^{-} ILCP, which also express *Tbx21*, were ILC1 primed and expressed T-bet by protein. Although both ILC1 and ILC2 primed ILCP had a great propensity to give rise to colonies of the respective ILC type in single-cell cultures, both retained some multi-potentcy, showing that these early ILC1 and ILC2 precursors retain a level of plasticity, reflecting the mixed ILC lineage effector programs in some of these cells.

Importantly ILC3 primed ILCP were distinct from LTiP in the fetal liver, which do not express PLZF, but did express RORγt, CXCR5, CCR6 and CD4, all markers associated with mature LTi. PLZF in the fetal liver therefore is the defining and distinguishing marker

between ILCP and LTiP.

Interestingly all $CXCR5^+$ ILCP have a mixed ILC1/ILC3 potential, suggesting that these lineages are developmentally more closely related than either to ILC2. In fact the transcriptional profile of single ILCP shows a greater overlap between the ILC1 and ILC3 programs compared to ILC2 primed precursors. Most of ILC1 and ILC3 primed ILCP express *CXCR5* and *Il2rb* and about half of ILC1 primed cells expressed *Rorc* in addition to *Tbx21*[55]. As discussed above, studies of Runx3 also have shown that both ILC1 and ILC3 share a requirement for Runx3, while ILC2 do not. As ILC3 show considerable plasticity and can give rise to ILC1 in the periphery[34], it is conceivable that ILC1 develop through the differentiation of ILC3. Based on fate-mapping studies in the periphery, ILC1s consist of both RORγt fate-mapped and non fate-mapped cells[59], suggesting that most likely ILC1s are generated by two independent pathways, a committed ILC1 precursor as well as through ILC3 plasticity, and it is likely that both of these processes already occur at the ILCP stage.

ILCP show a considerable degree of heterogeneity, which can be attributed to the rapid differentiation of these precursors into lineage-primed ILCs. Interestingly this process occurs through multi-lineage priming, where ILCP show evidence of mixed ILC effector programs before the resolution into distinct lineages. The ILCP therefore is the stage of ILC lineage trifurcation and we have identified the earliest ILC1, 2 and 3 precursors and their transcriptional profiles.

4.1.1.4 Cross Regulation of ILCP Differentiation into ILC1s, ILC2s, and ILC3s

As discussed above, the generation of ILC1, ILC2, and ILC3 lineages is initiated at the ILCP stage and proceeds through the trifurcation of multilineage primed precursors, as they undergo several rounds of cell division. This developmental trifurcation is reminiscent of the NKT thymic precursor differentiating into NKT1, NKT2, and NKT17 lineages[183]

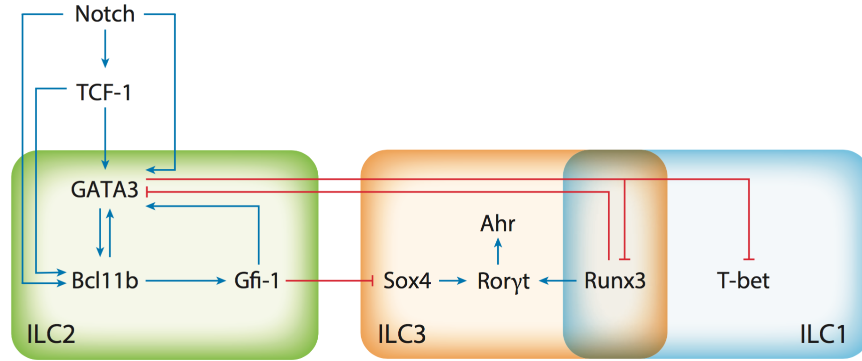

Figure 4.2: Transcription factor networks regulating cytokine effector programs in innate lymphoid cell (ILC) lineages. Blue arrows depict positive interactions, whereas red lines depict inhibitory interactions at the gene or protein level.
Figure is adapted from ref. 50

(**Fig.4.3**), because it is also immediately preceded by the induction of high amounts of PLZF and rounds of cell division and is independent of exogenous polarizing cytokines and STAT signaling associated with the differentiation of T_H1, T_H2, and T_H17 cells. Ectopic expression of PLZF using a *Cd4* promoter is sufficient to transform naive CD4 thymocytes into effector-type cells that downregulate CD62L, upregulate CD44 and ICOS, and can produce both IL-4 and IFN-γ[103,184]. Conversely, in PLZF-deficient mice, NKT cells revert to a naive phenotype, implying that PLZF plays a crucial role in directing the acquisition of effector programs during lymphocyte development[103,156,157]. Notably, PLZF deficiency also alters expression of ICOS and CD62L in ILC2s and markedly impairs their ability to produce IL-5 and IL-13 in response to IL-33 or ionomycin and PMA or to mediate allergic airway inflammation after papain inhalation or infection with Nippostrongylus brasiliensis[60,104]. In fact, PLZF directly can regulate *Rorc*, *Runx1*, *Rora*, *Icos*, *Il4* and *Il13* and has been shown to suppress Bach2 expression, which is a negative regulator of T helper effector programs[105]. These observations suggest that PLZF plays a similar role in several aspects of ILCP and NKT cell differentiation.

Other signals critically regulate the polarization of ILC lineages. For example, a Notch signal seems required for the development of ILC2s, possibly through a cascade involving

Tcf7, *Gata3*, *Bcl11b*, and *Gfi1* [18,96,119,120] (**Fig.4.3**). Notch signaling is also important for peripheral NCR^{+}ILC3s, but this may be a peripheral event dependent on aryl hydrocarbon receptor signaling and T-bet [35,133]. Conversely, dietary retinoic acid is required for the development of LTis in vivo, likely through direct binding of RXRs and RARs to *Rorc* [46,140]. Retinoic acid appears to inhibit the generation of ILC2s as well, in part through limiting the expansion of the immature bone marrow ILC2s [46]. Although LTis were not always distinguished from ILC3s in these studies, the effects of retinoic acid seem to affect both populations, suggesting a common mechanism.

In summary, multiple genetic and environmental factors modulate the output of ILC1s, ILC2s, and ILC3s, with potentially significant health implications.

In conclusion, we have provided a blueprint of ILC development, which was confirmed by functional transfer studies, and a hierarchy of transcriptional regulators that act at different ILC developmental stages.

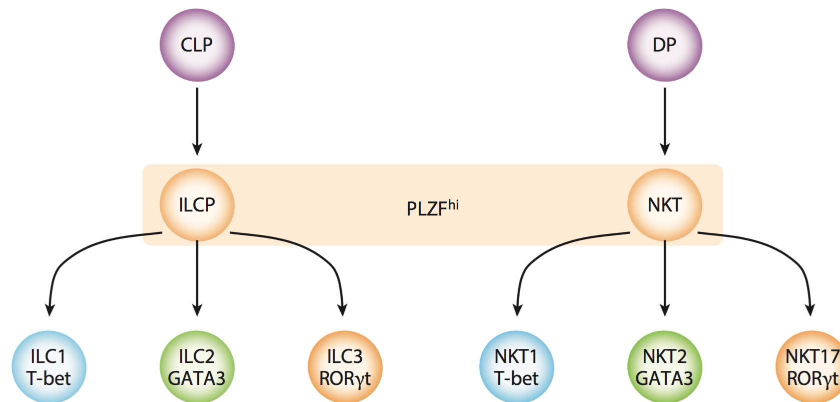

Figure 4.3: Developmental acquisition of effector cytokine programs by ILC and natural killer T cell (NKT) precursors. The trifurcation of ILC and NKT precursors into polarized effector lineages is preceded by induction of PLZF. Abbreviation: CLP, common lymphoid progenitor; DP, double-positive thymocytes. Figure is adapted from ref. 50

4.2 Future Directions

4.2.1 Developmental Placement of the EILP and CHILP

Due to its lack of IL-7Rα chain expression the EILP has eluded earlier studies of ILC precursors and makes it hard to place the EILP within the existing developmental scheme for ILCs.

Based on its expression of *Tcf7* and low levels of *Id2*, it is likely that the EILP developmentally follows the αLP, which does not express *Tcf7*, unlike LTiP and ILCP. The puzzling observation about the EILP is however that it does not express the IL-7Rα chain, whereas all other known ILC precursors including the CLP express it. This suggests that the EILP either represents an alternative pathway for ILC development, that does not depend on the CLP, or that an earlier precursor, potentially the αLP, transiently downregulates the IL-7 receptor to give rise to the EILP.

In fact there is precedent to assume that the IL-7Rα chain could transiently be downregulated during ILC development. ETPs have been shown to be marked by IL-7R fate mapping, but do not express *Il7ra* mRNA or protein[163,185]. Similarly double positive (DP) thymocytes appear to transiently downregulate *Il7ra* expression, which is restored upon positive selection[186]. It therefore remains to be determined whether perhaps early precursors with ILC, cNK and LTi potential, such as the αLP transiently downregulate the IL-7Rα chain to give rise to the EILP. Similarly it is not clear whether the EILP in turn does upregulate the IL-7Rα chain to give rise to the CHILP, LTiP and ILCP. These changes assume that ILC development proceeds in a linear fashion from the CLP. Whether changes in the expression of the IL-7Rα chain take place in EILP or αLP can only be investigated by observing pure populations in culture over time, to assess their capability to give rise to each other and the ILCP.

It is also not certain whether the EILP in fact derives from the CLP as assumed. This

issue could be addressed through fate-mapping of the IL-7Rα chain, which is expressed at the CLP stage. EILP should therefore be fate-mapped if they in fact derive from the CLP. This approach would also clarify whether the EILP represents an alternative route for ILC development as compared to the developmental path that depends on the CLP and subsequent IL-7Rα^{+} precursors.

Another interesting question is how the IL-7Rα chain is potentially downregulated during ILC development. Studies in B and T cell development have shown that Runx1 can in fact induce the expression of the IL-7Rα chain[187,188]. However whether Runx1 is the transcription factor required for the upregulation of the IL-7Rα chain in EILP and whether it is expressed at the EILP stage, remains to be determined. It is also not clear whether strong IL-7 signaling at the αLP stage can trigger the transient downregulation of the IL-7Rα chain. If this were the case, it also raises the question as to the precise niche where ILC precursors could receive such a signal in the fetal liver or the bone marrow.

Chea and colleagues also have shown that *Id2* expression levels can be used to map a developmental progression of ILC precursors, with low levels of *Id2* expression in the Flt3$^{+}\alpha$LP and intermediate and high levels in the Flt3$^{-}\alpha$LP and LTiP/ILCP respectively[98]. Assessing *Tcf7* and *Id2* expression at the same time in *Tcf7*^{GFP}*Id2*^{YFP} double transgenic mice could be informative about the precise level of *Id2* expression in the EILP as compared to other precursors and allow for a better placement of the EILP.

However a definitive way to place the EILP within the current scheme of ILC development would be an extensive analysis of its expression pattern on a single-cell level. Tracking the continuity of transcriptional profiles of EILPs within the context of other ILC precursors will tell where the EILP can be placed and perhaps reveal transitional stages among this population.

The other ILC precursor population that requires further study is the CHILP. The original description of the CHILP relied on the expression of high levels of Id2 among Lin^{-}IL-

$7R\alpha^+\alpha4\beta7^+CD25^-$ cells in the bone marrow. This population however is contaminated by PLZF expressing ILCP and would also encompass the LTi in the fetal liver[59]. Therefore the precise identity of an ILC precursor upstream of the ILCP and the LTiP that would fit the description of the CHILP remains unclear. The CHILP could be a late stage of the αLP, that is $PLZF^-$, $ROR\gamma t^-$, expresses high levels of Id2 and Tcf-1. The CHILP therefore most likely corresponds to a transitional cluster of cells between the αLP and the ILCP and studies of $Tcf7^{GFP}Id2^{YFP}$ double transgenic reporter mice could further elucidate the identity of the CHILP. The isolation and evaluation of the CHILP could lead to further insight on the bifurcation of ILCP and LTiP, which possibly happens at this transitional stage.

In conclusion, a better definition and a precise placement of the EILP and the CHILP will allow for the characterization of expression and epigenetic changes between stages of ILC development and also allow for the inference of new mechanisms that control ILC developmental progression. Specifically the control of *Tcf7* expression.

4.2.2 The Regulation of Tcf7 expression and the Emergence of the EILP

Tcf-1 is the hallmark transcription factor in the EILP and has been shown to be required for the development of all ILC lineages[57,95,96]. Its expression also marks the transition from αLP to the ILCP and LTiP stage. However it is not clear how *Tcf7* expression is regulated during ILC development.

Notch signaling, as a known inducer of *Tcf7* expression in T cells, has been suggested to play a role in ILC development by upregulating *Tcf7*[57]. It has also been suggested that Notch signaling augments the upregulation of the defining marker of all ILC precursors, $\alpha4\beta7$, on CLP[56]. However Notch signaling is not required for the development of ILCs or $\alpha4\beta7$ upregulation, although it can influence the function of LTi and ILC3s in the periphery[40,98]. Deletion of the DNA binding adapter *Rbpj* did not have any influence on the expression of key ILC developmental transcription factors, *Id2*, *Tox*, *Gata3* and *Tcf7* itself.

Since Notch does not appear to regulate the expression of *Tcf7* during ILC development, it is possible that Wnt/β-catenin signaling may be involved in ILC development, as it is also a regulator of *Tcf7*. However it has not been formally investigated whether the Wnt/β-catenin signaling pathway plays a role in ILC development.

Although many key players in ILC development as well as ILC developmental stages have been identified, the regulation of transcription factors and their interactions with each other pose an intriguing area for further study. The assessment of the epigenetic landscape of EILPs, which are potentially the earliest stage of *Tcf7* expression in ILC development, could elucidate how *Tcf7* expression is regulated during ILC development.

4.2.3 Sox4 and Runx1 in ILC Development

We have shown that Sox4 and Runx1 are transiently expressed at a transitional stage between αLP and ILCP, suggesting that both factors may function in the bifurcation of LTi and ILCP, however their roles in ILC development are not well understood.

Runx1 (also AML1) is a family member of the Drosophila Runt-related transcription factors, which consists of, Runx1, Runx2 and Runx3. Runx proteins heterodimerize with the Core binding factor β (Cbfβ) for efficient DNA binding. Runx proteins function in the immune system as well as neuronal development and osteoblast formation.

Runx1 has been shown to play a role in B cells development and T_H17 differentiation[187,189–191]. Interestingly Runx1 was also capable of positively regulating the expression of *Id2*, *Ets1* and *Sox4*, which all are upregulated at the transitional cluster B stage. Although *Id2* is already expressed earlier, it is in fact further upregulated with a slight delay compared to Sox4 and Runx1, which are already starting to be expressed at a late αLP stage.

Combined This data suggests that Runx1 potentially induces Id2 at the transitional stage, although it does not trigger its expression at the onset of ILC development. The induction of Id2 at the transitional stage also is paired with a further reduction in T cell

generating potential in ILCP[55], showing that the upregulation of Id2 is essential to further repress non-ILC lineages.

Other studies in T cells have also shown that Runx1 positively influences RORγt expression and negatively regulates Gata3 expression, thereby promoting T_H17 differentiation. Runx1 in turn appears to also be negatively regulated by T-bet[189]. In fact mice that lack Runx1 or conditional Cbfβ knockout mice show impaired development of secondary lymphoid tissues and show a severe reduction in LTi precursors[144], which depend on RORγt for their development.

The SRY-related high-mobility-group-box (Sox) protein Sox4 is capable of upregulating *Rorc*, *Il17* expression and represses Gata3 in $\gamma\delta$T cells as well as T_H17 cells[97,192]. Sox4 is capable of directly binding and represses Gata3 function at the protein level, preventing T_H2 differentiation[192]

Together, Runx1 and Sox4 are potent inducers of RORγt and the T_H17 effector program. Both factors also repress Gata3 and thereby the T_H2 effector program. It is likely that both mechanisms also act at the junction of LTi and ILC, when Sox4 and Runx1 are highly expressed, to promote the development of the LTiP. Studies of both Runx1 and Sox4 knockout mice would elucidate the role of both transcription factors in ILC development and potentially answer the question as to how the bifurcation of LTi and ILC occurs.

4.3 Conclusion

Using the newly identified ILCP as a focal point in our studies, we have clarified the stages and the sequence of transcription factors driving ILC development. Over 15 transcription factors, many of which are also active in T cell development, combine to regulate the emergence of ILCs from early lymphoid precursors and their differentiation into polarized lineages.

We identified an early ILC developmental transcription factor network of Id2, Nfil3 and Tox, that acts at the αLP stage to promote ILC development and at the same time prevent the development of the adaptive arm of the immune system. The expression of *Zbtb16*, *Tcf7*, *Sox4* and *Runx1* marked a transitional cluster of precursors that clearly showed cells biased to either the ILC or the LTi lineage, identifying this cluster as the stage of bifurcation of LTiP and ILCP and showing that LTi and ILC3 indeed develop through different pathways.

We also showed that the ILCP is the stage of ILC lineage trifurcation and identified transcriptional modules that act at the ILCP stage to define ILC differentiation, a process which occurs through multi-lineage priming and the presence of mixed ILC modules in early ILCP.

In summary we have established a blue-print of ILC development with a refined sequence of transcription factor expression. This study gives insight into stage specific transcriptional networks and potential interactions that drive ILC development and differentiation.

Further studies of additional precursors, such as the EILP, the CHILP and the developmental transitions between stage specific transcription factors and their individual and collective functions is the next challenge. These fundamental studies will provide further insights into mechanisms of diseases as well as opportunities for therapeutic manipulation of ILCs.

REFERENCES

[1] David Artis and Hergen Spits. The biology of innate lymphoid cells. *Nature*, 517(7534):293–301, January 2015.

[2] Yan Zheng, Patricia A Valdez, Dimitry M Danilenko, Yan Hu, Susan M Sa, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nature Medicine*, 14(3):282–289, March 2008.

[3] Qi Yang, Steven A Saenz, Daniel A Zlotoff, David Artis, and Avinash Bhandoola. Cutting edge: Natural helper cells derive from lymphoid progenitors. *The Journal of Immunology*, 187(11):5505–5509, December 2011.

[4] R E Mebius, T Miyamoto, J Christensen, J Domen, T Cupedo, et al. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45(+)CD4(+)CD3(-) cells, as well as macrophages. *Journal of immunology (Baltimore, Md. : 1950)*, 166(11):6593–6601, 2001.

[5] Hergen Spits, David Artis, Marco Colonna, Andreas Diefenbach, James P Di Santo, et al. Innate lymphoid cells–a proposal for uniform nomenclature. *Nature reviews. Immunology*, 13(2):145–149, February 2013.

[6] Cécile Daussy, Fabrice Faure, Katia Mayol, Sébastien Viel, Georg Gasteiger, et al. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. *Journal of Experimental Medicine*, 211(3):563–577, March 2014.

[7] Scott M Gordon, Julie Chaix, Levi J Rupp, Junmin Wu, Sharline Madera, et al. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity*, 36(1):55–67, January 2012.

[8] Giuseppe Sciumè, Kiyoshi Hirahara, Hayato Takahashi, Arian Laurence, Alejandro V Villarino, et al. Distinct requirements for T-bet in gut innate lymphoid cells. *Journal of Experimental Medicine*, 209(13):2331–2338, December 2012.

[9] Dorothy K Sojka, Beatrice Plougastel-Douglas, Liping Yang, Melissa A Pak-Wittel, Maxim N Artyomov, et al. Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *eLife*, 3:e01659, 2014.

[10] Olga Pikovskaya, Julie Chaix, Nyanza J Rothman, Amélie Collins, Yen-Hua Chen, et al. Cutting Edge: Eomesodermin Is Sufficient To Direct Type 1 Innate Lymphocyte Development into the Conventional NK Lineage. *The Journal of Immunology*, 196(4):1449–1454, February 2016.

[11] Jochem H Bernink, Charlotte P Peters, Marius Munneke, Anje A Te Velde, Sybren L Meijer, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nature Immunology*, 14(3):221–229, March 2013.

[12] Anja Fuchs, William Vermi, Jacob S Lee, Silvia Lonardi, Susan Gilfillan, et al. Intraepithelial Type 1 Innate Lymphoid Cells Are a Unique Subset of IL-12- and IL-15-Responsive IFN-γ-Producing Cells. *Immunity*, 38(4):769–781, April 2013.

[13] Hui Peng, Xiaojun Jiang, Yonglin Chen, Dorothy K Sojka, Haiming Wei, et al. Liver-resident NK cells confer adaptive immunity in skin-contact inflammation. *Journal of Clinical Investigation*, 123(4):1444–1456, April 2013.

[14] Michael G Constantinides, Herman Gudjonson, Benjamin D McDonald, Isabel E Ishizuka, Philip A Verhoef, et al. PLZF expression maps the early stages of ILC1 lineage development. *Proceedings of the National Academy of Sciences*, 112(16):5123–5128, April 2015.

[15] John W Fathman, Deepta Bhattacharya, Matthew A Inlay, Jun Seita, Holger Karsunky, et al. Identification of the earliest natural killer cell-committed progenitor in murine bone marrow. *Blood*, 118(20):5439–5447, November 2011.

[16] Sebastian Carotta, Swee Heng Milon Pang, Stephen L Nutt, and Gabrielle T Belz. Identification of the earliest NK-cell precursor in the mouse BM. *Blood*, 117(20):5449–5452, May 2011.

[17] Kazuyo Moro, Taketo Yamada, Masanobu Tanabe, Tsutomu Takeuchi, Tomokatsu Ikawa, et al. Innate production of TH2 cytokines by adipose tissue-associated c-Kit+Sca-1+ lymphoid cells. *Nature*, 463(7280):540–544, December 2009.

[18] Daniel R Neill, See Heng Wong, Agustin Bellosi, Robin J Flynn, Maria Daly, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*, 464(7293):1367–1370, April 2010.

[19] David Voehringer, Tiffany A Reese, Xiaozhu Huang, Kanade Shinkai, and Richard M Locksley. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *The Journal of experimental medicine*, 203(6):1435–1446, June 2006.

[20] Timotheus Y F Halim, Ramona H Krauß, Ann C Sun, and Fumio Takei. Lung Natural Helper Cells Are a Critical Source of Th2 Cell-Type Cytokines in Protease Allergen-Induced Airway Inflammation. *Immunity*, 36(3):451–463, February 2012.

[21] Jesse C Nussbaum, Steven J Van Dyken, Jakob von Moltke, Laurence E Cheng, Alexander Mohapatra, et al. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature*, pages 1–13, September 2013.

[22] Laurel A Monticelli, Gregory F Sonnenberg, Michael C Abt, Theresa Alenghat, Carly G K Ziegler, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nature Immunology*, 12(11):1045–U47, November 2011.

[23] Hiroki Kabata, Kazuyo Moro, Koichi Fukunaga, Yusuke Suzuki, Jun Miyata, et al. Thymic stromal lymphopoietin induces corticosteroid resistance in natural helper cells during airway inflammation. *Nature communications*, 4:2675, 2013.

[24] B S Kim, M C Siracusa, S A Saenz, M Noti, and L A Monticelli. TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. Sci Transl Med. 2013; 5 (23363980): 170.

[25] Timotheus Y F Halim, Aric MacLaren, Mark T Romanish, Matthew J Gold, Kelly M McNagny, et al. Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. *Immunity*, 37(3):463–474, September 2012.

[26] Jonathan R Brestoff, Brian S Kim, Steven A Saenz, Rachel R Stine, Laurel A Monticelli, et al. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature*, 519(7542):242–246, December 2014.

[27] Min-Woo Lee, Justin I Odegaard, Lata Mukundan, Yifu Qiu, Ari B Molofsky, et al. Activated Type 2 Innate Lymphoid Cells Regulate Beige Fat Biogenesis. *Cell*, 160(1-2):74–87, December 2014.

[28] Timothy E O'Sullivan, Moritz Rapp, Xiying Fan, Orr-El Weizman, Priya Bhardwaj, et al. Adipose-Resident Group 1 Innate Lymphoid Cells Promote Obesity-Associated Insulin Resistance. *Immunity*, 45(2):428–441, August 2016.

[29] Ju Qiu, Jennifer J Heller, Xiaohuan Guo, Zong-ming E Chen, Kamonwan Fish, et al. The Aryl Hydrocarbon Receptor Regulates Gut Immunity through Modulation of Innate Lymphoid Cells. *Immunity*, 36(1):92–104, January 2012.

[30] Marina Cella, Anja Fuchs, William Vermi, Fabio Facchetti, Karel Otero, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature*, 457(7230):722–725, February 2009.

[31] Hiroaki Takatori, Yuka Kanno, Wendy T Watford, Cristina M Tato, Greta Weiss, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *Journal of Experimental Medicine*, 206(1):35–41, January 2009.

[32] Masayuki Tsuji, Keiichiro Suzuki, Hiroshi Kitamura, Mikako Maruya, Kazuo Kinoshita, et al. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin A generation in the gut. *Immunity*, 29(2):261–271, August 2008.

[33] A Lügering, M Ross, M Sieker, J Heidemann, I R Williams, et al. CCR6 identifies lymphoid tissue inducer cells within cryptopatches. *Clinical and experimental immunology*, 160(3):440–449, June 2010.

[34] Christoph S N Klose, Elina A Kiss, Vera Schwierzeck, Karolina Ebert, Thomas Hoyler, et al. A T-bet gradient controls the fate and function of CCR6-RORγt+ innate lymphoid cells. *Nature*, 494(7436):261–265, February 2013.

[35] Lucille C Rankin, Joanna R Groom, Michaël Chopin, Marco J Herold, Jennifer A Walker, et al. The transcription factor T-bet is essential for the development of NKp46+ innate lymphocytes via the Notch pathway. *Nature Immunology*, 14(4):389–395, April 2013.

[36] Ana Reynders, Nadia Yessaad, Thien-Phong Vu Manh, Marc Dalod, Aurore Fenis, et al. Identity, regulation and in vivo function of gut NKp46+RORγt+ and NKp46+RORγt- lymphoid cells. *The EMBO Journal*, 30(14):2934–2947, July 2011.

[37] Shinichiro Sawa, Matthias Lochner, Naoko Satoh-Takayama, Sophie Dulauroy, Marion Bérard, et al. RORγt+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nature Immunology*, 12(4):320–326, April 2011.

[38] Gregory F Sonnenberg and David Artis. Innate Lymphoid Cell Interactions with Microbiota: Implications for Intestinal Health and Disease. *Immunity*, 37(4):601–610, October 2012.

[39] Chao Zhong, Kairong Cui, Christoph Wilhelm, Gangqing Hu, Kairui Mao, et al. Group 3 innate lymphoid cells continuously require the transcription factor GATA-3 after commitment. *Nature Immunology*, 17(2):169–178, November 2015.

[40] Sylvestre Chea, Thibaut Perchet, Maxime Petit, Thomas Verrier, Delphine Guy-Grand, et al. Notch signaling in group 3 innate lymphoid cells modulates their plasticity. *Science signaling*, 9(426):ra45–ra45, May 2016.

[41] Lucille C Rankin, Mathilde J H Girard-Madoux, Cyril Seillet, Lisa A Mielke, Yann Kerdiles, et al. Complementarity and redundancy of IL-22-producing innate lymphoid cells. *Nature Immunology*, 17(2):179–186, November 2015.

[42] Naoko Satoh-Takayama, Nicolas Serafini, Thomas Verrier, Abdessalem Rekiki, Jean Christophe Renauld, et al. The Chemokine Receptor CXCR6 Controls the Functional Topography of Interleukin-22 Producing Intestinal Innate Lymphoid Cells. *Immunity*, pages 1–13, November 2014.

[43] Yuefeng Huang, Liying Guo, Jin Qiu, Xi Chen, Jane Hu-Li, et al. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nature Immunology*, December 2014.

[44] Yoichiro Ohne, Jonathan S Silver, LuAnn Thompson-Snipes, Magalie A Collet, Jean Philippe Blanck, et al. IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. *Nature Immunology*, 17(6):646–655, June 2016.

[45] Jochem H Bernink, Lisette Krabbendam, Kristine Germar, Esther de Jong, Konrad Gronke, et al. Interleukin-12 and -23 Control Plasticity of CD127(+) Group 1 and Group 3 Innate Lymphoid Cells in the Intestinal Lamina Propria. *Immunity*, 43(1):146–160, July 2015.

[46] S P Spencer, C Wilhelm, Q Yang, J A Hall, N Bouladoux, et al. Adaptation of innate lymphoid cells to a micronutrient deficiency promotes type 2 barrier immunity. *Science*, 343(6169):432–437, January 2014.

[47] Frédéric Vély, Vincent Barlogis, Blandine Vallentin, Bénédicte Neven, Christelle Piperoglou, et al. Evidence of innate lymphoid cell redundancy in humans. *Nature Immunology*, 17(11):1291–1299, November 2016.

[48] Georg Gasteiger, Xiying Fan, Stanislav Dikiy, Sue Y Lee, and Alexander Y Rudensky. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science*, 350(6263):981–985, November 2015.

[49] Henrik N Kløverpris, Samuel W Kazer, Jenny Mjösberg, Jenniffer M Mabuka, Amanda Wellmann, et al. Innate Lymphoid Cells Are Depleted Irreversibly during Acute HIV-1 Infection in the Absence of Viral Suppression. *Immunity*, 44(2):391–405, February 2016.

[50] Isabel E Ishizuka, Michael G Constantinides, Herman Gudjonson, and Albert Bendelac. The Innate Lymphoid Cell Precursor. *Annual review of immunology*, 34:299–316, May 2016.

[51] Gerard Eberl, Shana Marmon, Mary-Jean Sunshine, Paul D Rennert, Yongwon Choi, et al. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nature Immunology*, 5(1):64–73, January 2004.

[52] H Yoshida, H Kawamoto, S M Santee, H Hashi, K Honda, et al. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. *Journal of immunology (Baltimore, Md. : 1950)*, 167(5):2511–2521, 2001.

[53] Shinichiro Sawa, Marie Cherrier, Matthias Lochner, Naoko Satoh-Takayama, Hans JOrg Fehling, et al. Lineage relationship analysis of RORgammat+ innate lymphoid cells. *Science*, 330(6004):665–669, October 2010.

[54] Cécilie Possot, Sandrine Schmutz, Sylvestre Chea, Laurent Boucontet, Anne Louise, et al. Notch signaling is necessary for adult, but not fetal, development of RORγt+ innate lymphoid cells. *Nature Immunology*, 12(10):949–958, September 2011.

[55] Isabel E Ishizuka, Sylvestre Chea, Herman Gudjonson, Michael G Constantinides, Aaron R Dinner, et al. Single-cell analysis defines the divergence between the innate lymphoid cell lineage and lymphoid tissue-inducer cell lineage. *Nature Immunology*, 17(3):269–276, March 2016.

[56] Marie Cherrier, Shinichiro Sawa, and Gerard Eberl. Notch, Id2, and RORγt sequentially orchestrate the fetal development of lymphoid tissue inducer cells. *Journal of Experimental Medicine*, 209(4):729–740, April 2012.

[57] Qi Yang, Fengyin Li, Christelle Harly, Shaojun Xing, Longyun Ye, et al. TCF-1 upregulation identifies early innate lymphoid progenitors in the bone marrow. *Nature Immunology*, 16(10):1044–1050, October 2015.

[58] Xiaofei Yu, Yuhao Wang, Mi Deng, Yun Li, Kelly A Ruhn, et al. The basic leucine zipper transcription factor NFIL3 directs the development of a common innate lymphoid cell precursor. *eLife*, 3, 2014.

[59] Christoph S N Klose, Melanie Flach, Luisa Möhle, Leif Rogell, Thomas Hoyler, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell*, 157(2):340–356, April 2014.

[60] Michael G Constantinides, Benjamin D McDonald, Philip A Verhoef, and Albert Bendelac. A committed precursor to innate lymphoid cells. *Nature*, 508(7496):397–401, April 2014.

[61] Y Yokota, A Mansouri, S Mori, S Sugawara, S Adachi, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature*, 397(6721):702–706, February 1999.

[62] Sandrine Schmutz, Nabil Bosco, Stéphane Chappaz, Onur Boyman, Hans Acha-Orbea, et al. Cutting edge: IL-7 regulates the peripheral pool of adult ROR gamma+ lymphoid tissue inducer cells. *The Journal of Immunology*, 183(4):2217–2221, August 2009.

[63] Naoko Satoh-Takayama, Sarah Lesjean-Pottier, Paulo Vieira, Shinichiro Sawa, Gerard Eberl, et al. IL-7 and IL-15 independently program the differentiation of intestinal CD3-NKp46+ cell subsets from Id2-dependent precursors. *Journal of Experimental Medicine*, 207(2):273–280, February 2010.

[64] Jennifer K Bando, Jesse C Nussbaum, Hong-Erh Liang, and Richard M Locksley. Type 2 innate lymphoid cells constitutively express arginase-I in the naive and inflamed lung. *Journal of Leukocyte Biology*, 94(5):877–884, November 2013.

[65] Laurel A Monticelli, Michael D Buck, Anne-Laure Flamar, Steven A Saenz, Elia D Tait Wojno, et al. Arginase 1 is an innate lymphoid-cell-intrinsic metabolic checkpoint controlling type 2 inflammation. *Nature Immunology*, 17(6):656–665, April 2016.

[66] Jennifer K Bando, Hong-Erh Liang, and Richard M Locksley. Identification and distribution of developing innate lymphoid cells in the fetal mouse intestine. *Nature Immunology*, 16(2):153–160, February 2015.

[67] Barbara L Kee. E and ID proteins branch out. 9(3):175–184, March 2009.

[68] Tomokatsu Ikawa, Hiroshi Kawamoto, Ananda W Goldrath, and Cornelis Murre. E proteins and Notch signaling cooperate to promote T cell lineage specification and commitment. *The Journal of experimental medicine*, 203(5):1329–1342, May 2006.

[69] J-K Kim, M Takeuchi, and Y Yokota. Impairment of intestinal intraepithelial lymphocytes in Id2 deficient mice. *Gut*, 53(4):480–486, 2004.

[70] Ana Pereira de Sousa, Claire Berthault, Alessandra Granato, Sheila Dias, Cyrille Ramond, et al. Inhibitors of DNA binding proteins restrict T cell potential by repressing Notch1 expression in Flt3-negative common lymphoid progenitors. *The Journal of Immunology*, 189(8):3822–3830, October 2012.

[71] M Ji, H Li, H C Suh, K D Klarmann, Y Yokota, et al. Id2 intrinsically regulates lymphoid and erythroid development via interaction with different target proteins. *Blood*, 112(4):1068–1077, May 2008.

[72] X H Sun. Constitutive expression of the Id1 gene impairs mouse B cell development. *Cell*, 79(5):893–900, December 1994.

[73] D Kim, X C Peng, and X H Sun. Massive apoptosis of thymocytes in T-cell-deficient Id1 transgenic mice. *Molecular and Cellular Biology*, 19(12):8240–8253, December 1999.

[74] M H Heemskerk, B Blom, G Nolan, A P Stegmann, A Q Bakker, et al. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *The Journal of experimental medicine*, 186(9):1597–1602, November 1997.

[75] A C Jaleco, A P Stegmann, M H Heemskerk, F Couwenberg, A Q Bakker, et al. Genetic modification of human B-cell development: B-cell development is inhibited by the dominant negative helix loop helix factor Id3. *Blood*, 94(8):2637–2646, October 1999.

[76] M D Boos, Y Yokota, G Eberl, and B L Kee. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. *Journal of Experimental Medicine*, 204(5):1119–1130, May 2007.

[77] Ian G Cowell. E4BP4/NFIL3, a PAR-related bZIP factor with many roles. *BioEssays*, 24(11):1023–1029, October 2002.

[78] Xiaofei Yu, Darcy Rollins, Kelly A Ruhn, Jeremy J Stubblefield, Carla B Green, et al. TH17 cell differentiation is regulated by the circadian clock. *Science*, 342(6159):727–730, November 2013.

[79] S Kamizono, G S Duncan, and M G Seidel. Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. *The Journal of experimental medicine*, 206(13):2977–2986, 2009.

[80] Duncan M Gascoyne, Elaine Long, Henrique Veiga Fernandes, Jasper de Boer, Owen Williams, et al. The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. *Nature Immunology*, 10(10):1118–1124, October 2009.

[81] Stefania Crotta, Annita Gkioka, Victoria Male, João H Duarte, Sophia Davidson, et al. The Transcription Factor E4BP4 Is Not Required for Extramedullary Pathways of NK Cell Development. *The Journal of Immunology*, 192(6):2677–2688, March 2014.

[82] Cyril Seillet, Lucille C Rankin, Joanna R Groom, Lisa A Mielke, Julie Tellier, et al. Nfil3 is required for the development of all innate lymphoid cell subsets. *Journal of Experimental Medicine*, 211(9):1733–1740, August 2014.

[83] Theresa L Geiger, Michael C Abt, Georg Gasteiger, Matthew A Firth, Margaret H O'Connor, et al. Nfil3 is crucial for development of innate lymphoid cells and host protection against intestinal pathogens. *Journal of Experimental Medicine*, 211(9):1723–1731, August 2014.

[84] Wei Xu, Rita G Domingues, Diogo Fonseca Pereira, Manuela Ferreira, Hélder Ribeiro, et al. NFIL3 orchestrates the emergence of common helper innate lymphoid cell precursors. *Cell reports*, 10(12):2043–2054, March 2015.

[85] Victoria Male, Ilaria Nisoli, Duncan M Gascoyne, and Hugh J M Brady. E4BP4: an unexpected player in the immune response. *Trends in immunology*, 33(2):98–102, February 2012.

[86] P Aliahmad and J Kaye. Development of all CD4 T lineages requires nuclear factor TOX. *Journal of Experimental Medicine*, 205(1):245–256, January 2008.

[87] Parinaz Aliahmad, Brian de la Torre, and Jonathan Kaye. Shared dependence on the DNA-binding factor TOX for the development of lymphoid tissue–inducer cell and NK cell lineages. *Nature Immunology*, 11(10):945–952, September 2010.

[88] Corey R Seehus, Parinaz Aliahmad, Brian de la Torre, Iliyan D Iliev, Lindsay Spurka, et al. The development of innate lymphoid cells requires TOX-dependent generation of a common innate lymphoid cell progenitor. *Nature Immunology*, 16(6):599–608, June 2015.

[89] Thomas Hoyler, Christoph S N Klose, Abdallah Souabni, Adriana Turqueti-Neves, Dietmar Pfeifer, et al. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity*, 37(4):634–648, October 2012.

[90] Queenie P Vong, Wai-Hang Leung, Jim Houston, Ying Li, Barbara Rooney, et al. TOX2 regulates human natural killer cell development by controlling T-BET expression. *Blood*, 124(26):3905–3913, December 2014.

[91] S Verbeek, D Izon, F Hofhuis, E Robanus-Maandag, H te Riele, et al. An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature*, 374(6517):70–74, March 1995.

[92] Brittany Nicole Weber, Anthony Wei-Shine Chi, Alejandro Chavez, Yumi Yashiro-Ohtani, Qi Yang, et al. A critical role for TCF-1 in T-lineage specification and differentiation. *Nature*, 476(7358):63–68, August 2011.

[93] Kristine Germar, Marei Dose, Tassos Konstantinou, Jiangwen Zhang, Hongfang Wang, et al. T-cell factor 1 is a gatekeeper for T-cell specification in response to Notch signaling. *Proceedings of the National Academy of Sciences*, 108(50):20060–20065, December 2011.

[94] Farrah C Steinke, Shuyang Yu, Xinyuan Zhou, Bing He, Wenjing Yang, et al. TCF-1 and LEF-1 act upstream of Th-POK to promote the CD4+ T cell fate and interact with Runx3 to silence Cd4 in CD8+ T cells. *Nature Immunology*, 15(7):646–656, May 2014.

[95] Lisa A Mielke, Joanna R Groom, Lucille C Rankin, Cyril Seillet, Frederick Masson, et al. TCF-1 controls ILC2 and NKp46+RORγt+ innate lymphocyte differentiation and protection in intestinal inflammation. *The Journal of Immunology*, 191(8):4383–4391, October 2013.

[96] Qi Yang, Laurel A Monticelli, Steven A Saenz, Anthony Wei-Shine Chi, Gregory F Sonnenberg, et al. T cell factor 1 is required for group 2 innate lymphoid cell generation. *Immunity*, 38(4):694–704, April 2013.

[97] Nidhi Malhotra, Kavitha Narayan, Ok Hyun Cho, Katelyn E Sylvia, Catherine Yin, et al. A network of high-mobility group box transcription factors programs innate interleukin-17 production. *Immunity*, 38(4):681–693, April 2013.

[98] Sylvestre Chea, Sandrine Schmutz, Claire Berthault, Thibaut Perchet, Maxime Petit, et al. Single-Cell Gene Expression Analyses Reveal Heterogeneous Responsiveness of Fetal Innate Lymphoid Progenitors to Notch Signaling. *Cell reports*, 14(6):1500–1516, February 2016.

[99] J Zhu, H Yamane, and W E Paul. Differentiation of effector CD4 T cell populations. *Annual review of immunology*, 2010.

[100] Gang Wei, Brian J Abraham, Ryoji Yagi, Raja Jothi, Kairong Cui, et al. Genome-wide Analyses of Transcription Factor GATA3-Mediated Gene Regulation in Distinct T Cell Types. *Immunity*, 35(2):299–311, August 2011.

[101] Nicolas Serafini, Roel G J Klein Wolterink, Naoko Satoh-Takayama, Wei Xu, Christian A J Vosshenrich, et al. Gata3 drives development of RORγt+ group 3 innate lymphoid cells. *Journal of Experimental Medicine*, 211(2):199–208, February 2014.

[102] Ryoji Yagi, Chao Zhong, Daniel L Northrup, Fang Yu, Nicolas Bouladoux, et al. The Transcription Factor GATA3 Is Critical for the Development of All IL-7Ra-Expressing Innate Lymphoid Cells. *Immunity*, pages 1–11, March 2014.

[103] Adam K Savage, Michael G Constantinides, Jin Han, Damien Picard, Emmanuel Martin, et al. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity*, 29(3):391–403, September 2008.

[104] Philip A Verhoef, Michael G Constantinides, Benjamin D McDonald, Joseph F Urban, Anne I Sperling, et al. Intrinsic functional defects of type 2 innate lymphoid cells impair innate allergic inflammation in promyelocytic leukemia zinc finger (PLZF)-deficient mice. *The Journal of allergy and clinical immunology*, 137(2):591–600.e1, October 2015.

[105] Ai-ping Mao, Michael G Constantinides, Rebecca Mathew, Zhixiang Zuo, Xiaoting Chen, et al. Multiple layers of transcriptional regulation by PLZF in NKT-cell development. *Proceedings of the National Academy of Sciences*, 113(27):7602–7607, July 2016.

[106] Rahul Roychoudhuri, Kiyoshi Hirahara, Kambiz Mousavi, David Clever, Christopher A Klebanoff, et al. BACH2 represses effector programs to stabilize Treg-mediated immune homeostasis. *Nature*, pages 1–7, June 2013.

[107] Kevin Ramirez, Katherine J Chandler, Christina Spaulding, Sasan Zandi, Mikael Sigvardsson, et al. Gene deregulation and chronic activation in natural killer cells deficient in the transcription factor ETS1. *Immunity*, 36(6):921–932, June 2012.

[108] Erin C Zook, Kevin Ramirez, Xiaohuan Guo, Grant van der Voort, Mikael Sigvardsson, et al. The ETS1 transcription factor is required for the development and cytokine-induced expansion of ILC2. *Journal of Experimental Medicine*, 213(5):687–696, May 2016.

[109] DCC Voon, Y T Hor, and Y Ito. The RUNX complex: reaching beyond haematopoiesis into immunity. *Immunology*, 146(4):523–536, 2015.

[110] Amélie Collins, Dan R Littman, and Ichiro Taniuchi. RUNX proteins in transcription factor networks that regulate T-cell lineage choice. *Nature reviews. Immunology*, 9(2):106–115, February 2009.

[111] Takashi Ebihara, Christina Song, Stacy H Ryu, Beatrice Plougastel-Douglas, Liping Yang, et al. Runx3 specifies lineage commitment of innate lymphoid cells. *Nature Immunology*, 16(11):1124–1133, November 2015.

[112] Peter Parham, Paul J Norman, Laurent Abi-Rached, and Lisbeth A Guethlein. Human-specific evolution of killer cell immunoglobulin-like receptor recognition of major histocompatibility complex class I molecules. *Philosophical Transactions: Biological Sciences*, 367(1590):800–811, March 2012.

[113] Victoria Male, Ilaria Nisoli, Tomasz Kostrzewski, David S J Allan, James R Carlyle, et al. The transcription factor E4bp4/Nfil3 controls commitment to the NK lineage and directly regulates Eomes and Id2 expression. *Journal of Experimental Medicine*, 211(4):635–642, April 2014.

[114] Werner Held, Hans Clevers, and Rudolf Grosschedl. Redundant functions of TCF-1 and LEF-1 during T and NK cell development, but unique role of TCF-1 for Ly49 NK cell receptor acquisition. *European Journal of Immunology*, 33(5):1393–1398, May 2003.

[115] See Heng Wong, Jennifer A Walker, Helen E Jolin, Lesley F Drynan, Emily Hams, et al. Transcription factor RORα is critical for nuocyte development. *Nature Immunology*, 13(3):229–236, March 2012.

[116] Roel G J Klein Wolterink, Nicolas Serafini, Menno van Nimwegen, Christian A J Vosshenrich, Marjolein J W de Bruijn, et al. Essential, dose-dependent role for the transcription factor Gata3 in the development of IL-5+ and IL-13+ type 2 innate lymphoid cells. *Proceedings of the National Academy of Sciences*, 110(25):10240–10245, June 2013.

[117] Jennifer A Walker, Christopher J Oliphant, Alexandros Englezakis, Yong Yu, Simon Clare, et al. Bcl11b is essential for group 2 innate lymphoid cell development. *Journal of Experimental Medicine*, 212(6):875–882, June 2015.

[118] Yong Yu, Cui Wang, Simon Clare, Juexuan Wang, Song-Choon Lee, et al. The transcription factor Bcl11b is specifically expressed in group 2 innate lymphoid cells and is essential for their development. *Journal of Experimental Medicine*, 212(6):865–874, June 2015.

[119] Danielle Califano, Jonathan J Cho, Mohammad N Uddin, Kyle J Lorentsen, Qi Yang, et al. Transcription Factor Bcl11b Controls Identity and Function of Mature Type 2 Innate Lymphoid Cells. *Immunity*, 43(2):354–368, August 2015.

[120] Chauncey J Spooner, Justin Lesch, Donghong Yan, Aly A Khan, Alex Abbas, et al. Specification of type 2 innate lymphocytes by the transcriptional determinant Gfi1. *Nature Immunology*, 14(12):1229–1236, December 2013.

[121] Peng Li, Shannon Burke, Juexuan Wang, Xiongfeng Chen, Mariaestela Ortiz, et al. Reprogramming of T cells to natural killer-like cells upon Bcl11b deletion. *Science*, 329(5987):85–89, July 2010.

[122] Long Li, Mark Leid, and Ellen V Rothenberg. An early T cell lineage commitment checkpoint dependent on the transcription factor Bcl11b. *Science*, 329(5987):89–93, July 2010.

[123] Jeffrey Vanvalkenburgh, Diana I Albu, Chandra Bapanpally, Sarah Casanova, Danielle Califano, et al. Critical role of Bcl11b in suppressor function of T regulatory cells and prevention of inflammatory bowel disease. *Journal of Experimental Medicine*, 208(10):2069–2081, September 2011.

[124] Yong Yu, Jason C H Tsang, Cui Wang, Simon Clare, Juexuan Wang, et al. Single-cell RNA-seq identifies a PD-1(hi) ILC progenitor and defines its development pathway. *Nature*, 539(7627):102–106, September 2016.

[125] Julie S Yuan, Philaretos C Kousis, Sara Suliman, Ioana Visan, and Cynthia J Guidos. Functions of Notch Signaling in the Immune System: Consensus and Controversies. 28(1):343–365, March 2010.

[126] Arivazhagan Sambandam, Ivan Maillard, Valerie P Zediak, Lanwei Xu, Rachel M Gerstein, et al. Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nature Immunology*, 6(7):663–670, July 2005.

[127] Derk Amsen, Andrey Antov, Dragana Jankovic, Alan Sher, Freddy Radtke, et al. Direct Regulation of Gata3 Expression Determines the T Helper Differentiation Potential of Notch. *Immunity*, 27(1):89–99, July 2007.

[128] Terry C Fang, Yumi Yashiro-Ohtani, Cristina Del Bianco, Dawson M Knoblock, Stephen C Blacklow, et al. Notch Directly Regulates Gata3 Expression during T Helper 2 Cell Differentiation. *Immunity*, 27(1):100–110, July 2007.

[129] Will Bailis, Yumi Yashiro-Ohtani, Terry C Fang, Robin D Hatton, Casey T Weaver, et al. Notch simultaneously orchestrates multiple helper T cell programs independently of cytokine signals. *Immunity*, 39(1):148–159, July 2013.

[130] Fanny L Casado, Kameshwar P Singh, and Thomas A Gasiewicz. Blood Cells, Molecules, and Diseases. *Blood Cells, Molecules, and Diseases*, 44(4):199–206, April 2010.

[131] Elina A Kiss, Cedric Vonarbourg, Stefanie Kopfmann, Elias Hobeika, Daniela Finke, et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science*, 334(6062):1561–1565, December 2011.

[132] Jacob S Lee, Marina Cella, and Marco Colonna. AHR and the Transcriptional Regulation of Type-17/22 ILC. *Frontiers in immunology*, 3:10, 2012.

[133] Jacob S Lee, Marina Cella, Keely G McDonald, Cecilia Garlanda, Gregory D Kennedy, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nature Immunology*, 13(2):144–151, February 2012.

[134] Kyle A Murphy, Loredana Quadro, and Lori A White. The intersection between the aryl hydrocarbon receptor (AhR)- and retinoic acid-signaling pathways. *Vitamins and hormones*, 75:33–67, 2007.

[135] Jason A Hall, John R Grainger, Sean P Spencer, and Yasmine Belkaid. The role of retinoic acid in tolerance and immunity. *Immunity*, 35(1):13–22, July 2011.

[136] Alexandre Larange and Hilde Cheroutre. Retinoic Acid and Retinoic Acid Receptors as Pleiotropic Modulators of the Immune System. *Annual review of immunology*, 34:369–394, May 2016.

[137] Jason A Hall, Jennifer L Cannons, John R Grainger, Liliane M Dos Santos, Timothy W Hand, et al. Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. *Immunity*, 34(3):435–447, March 2011.

[138] Myung H Kim, Elizabeth J Taparowsky, and Chang H Kim. Retinoic Acid Differentially Regulates the Migration of Innate Lymphoid Cell Subsets to the Gut. *Immunity*, 43(1):107–119, June 2015.

[139] Lisa A Mielke, Sarah A Jones, Mathilde Raverdeau, Rowan Higgs, Anna Stefanska, et al. Retinoic acid expression associates with enhanced IL-22 production by $\gamma\delta$ T cells and innate lymphoid cells and attenuation of intestinal inflammation. *Journal of Experimental Medicine*, 210(6):1117–1124, June 2013.

[140] Serge A van de Pavert, Manuela Ferreira, Rita G Domingues, Hélder Ribeiro, Rosalie Molenaar, et al. Maternal retinoids control type 3 innate lymphoid cells and set the offspring immunity. *Nature*, 508(7494):123–127, April 2014.

[141] Andreas Diefenbach, Marco Colonna, and Shigeo Koyasu. Development, differentiation, and diversity of innate lymphoid cells. *Immunity*, 41(3):354–365, September 2014.

[142] Nicolas Serafini, Christian A J Vosshenrich, and James P Di Santo. Transcriptional regulation of innate lymphoid cell fate. *Nature reviews. Immunology*, 15(7):415–428, July 2015.

[143] Kazuyo Moro and Shigeo Koyasu. Innate lymphoid cells, possible interaction with microbiota. *Seminars in immunopathology*, 37(1):27–37, January 2015.

[144] Masashi Tachibana, Mari Tenno, Chieko Tezuka, Machiko Sugiyama, Hisahiro Yoshida, et al. Runx1/Cbfβ2 complexes are required for lymphoid tissue inducer cell differentiation at two developmental stages. *The Journal of Immunology*, 186(3):1450–1457, February 2011.

[145] Peter Laslo, Chauncey J Spooner, Aryeh Warmflash, David W Lancki, Hyun-Jun Lee, et al. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell*, 126(4):755–766, August 2006.

[146] M Hu, D Krause, M Greaves, S Sharkis, M Dexter, et al. Multilineage gene expression precedes commitment in the hemopoietic system. *Genes & Development*, 11(6):774–785, March 1997.

[147] Toshihiro Miyamoto, Hiromi Iwasaki, Boris Reizis, Min Ye, Thomas Graf, et al. Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Developmental Cell*, 3(1):137–147, July 2002.

[148] Samuel Yao-Ming Ng, Toshimi Yoshida, Jiangwen Zhang, and Katia Georgopoulos. Genome-wide lineage-specific transcriptional networks underscore Ikaros-dependent lymphoid priming in hematopoietic stem cells. *Immunity*, 30(4):493–507, April 2009.

[149] Yaron E Antebi, Shlomit Reich-Zeliger, Yuval Hart, Avi Mayo, Inbal Eizenberg, et al. Mapping differentiation under mixed culture conditions reveals a tunable continuum of T cell fates. *PLoS Biology*, 11(7):e1001616, July 2013.

[150] Bandar Ali Suliman, Dakang Xu, and Bryan Raymond George Williams. The promyelocytic leukemia zinc finger protein: two decades of molecular oncology. *Frontiers in oncology*, 2:74, 2012.

[151] Mathias Uhlén, Erik Björling, Charlotta Agaton, Cristina Al-Khalili Szigyarto, Bahram Amini, et al. A human protein atlas for normal and cancer tissues based on antibody proteomics. *Molecular & cellular proteomics : MCP*, 4(12):1920–1932, December 2005.

[152] Maria Barna, Taha Merghoub, José A Costoya, Davide Ruggero, Matthew Branford, et al. Plzf mediates transcriptional repression of HoxD gene expression through chromatin remodeling. *Developmental Cell*, 3(4):499–510, October 2002.

[153] M Barna, N Hawe, L Niswander, and P P Pandolfi. Plzf regulates limb and axial skeletal patterning. *Nature genetics*, 2000.

[154] José A Costoya, Robin M Hobbs, Maria Barna, Giorgio Cattoretti, Katia Manova, et al. Essential role of Plzf in maintenance of spermatogonial stem cells. *Nature genetics*, 36(6):653–659, June 2004.

[155] F William Buaas, Andrew L Kirsh, Manju Sharma, Derek J McLean, Jamie L Morris, et al. Plzf is required in adult male germ cells for stem cell self-renewal. *Nature genetics*, 36(6):647–652, June 2004.

[156] Adam K Savage, Michael G Constantinides, and Albert Bendelac. Promyelocytic leukemia zinc finger turns on the effector T cell program without requirement for agonist TCR signaling. *The Journal of Immunology*, 186(10):5801–5806, May 2011.

[157] Damian Kovalovsky, Olisambu U Uche, Sonia Eladad, Robin M Hobbs, Woelsung Yi, et al. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nature Immunology*, 9(9):1055–1064, September 2008.

[158] Michael P Seiler, Rebecca Mathew, Megan K Liszewski, Chauncey J Spooner, Chauncey Spooner, et al. Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling. *Nature Immunology*, 13(3):264–271, March 2012.

[159] Julia Raberger, Alexandra Schebesta, Shinya Sakaguchi, Nicole Boucheron, K Emelie M Blomberg, et al. The transcriptional regulator PLZF induces the development of CD44 high memory phenotype T cells. *Proceedings of the National Academy of Sciences*, 105(46):17919–17924, November 2008.

[160] Vivian M Turner, Sandra Gardam, and Robert Brink. Lineage-specific transgene expression in hematopoietic cells using a Cre-regulated retroviral vector. *Journal of Immunological Methods*, 360(1-2):162–166, August 2010.

[161] Bosiljka Tasic, Simon Hippenmeyer, Charlene Wang, Matthew Gamboa, Hui Zong, et al. Site-specific integrase-mediated transgenesis in mice via pronuclear injection. *Proceedings of the National Academy of Sciences of the United States of America*, 108(19):7902–7907, May 2011.

[162] R Shaknovich, P L Yeyati, S Ivins, A Melnick, C Lempert, et al. The promyelocytic leukemia zinc finger protein affects myeloid cell growth, differentiation, and apoptosis. *Molecular and Cellular Biology*, 18(9):5533–5545, September 1998.

[163] Susan M Schlenner, Vikas Madan, Katrin Busch, Annette Tietz, Carolin LAufle, et al. Fate Mapping Reveals Separate Origins of T Cells and Myeloid Lineages in the Thymus. *Immunity*, 32(3):426–436, March 2010.

[164] Parinaz Aliahmad, Akop Seksenyan, and Jonathan Kaye. The many roles of TOX in the immune system. *Current Opinion in Immunology*, 24(2):173–177, April 2012.

[165] P Aliahmad, A Kadavallore, B de la Torre, D Kappes, and J Kaye. TOX Is Required for Development of the CD4 T Cell Lineage Gene Program. *The Journal of Immunology*, 187(11):5931–5940, November 2011.

[166] Beverley Wilkinson, Jeff Y F Chen, Peggy Han, Kevin M Rufner, Olivia D Goularte, et al. TOX: an HMG box protein implicated in the regulation of thymocyte selection. *Nature Immunology*, 3(3):272–280, February 2002.

[167] Sonja Dittmer, Zsuzsa Kovacs, Shauna H Yuan, Gabriella Siszler, Manfred Kögl, et al. TOX3 is a neuronal survival factor that induces transcription depending on the presence of CITED1 or phosphorylated CREB in the transcriptionally active complex. *Journal of cell science*, 124(Pt 2):252–260, January 2011.

[168] Douglas F Easton, Karen A Pooley, Alison M Dunning, Paul D P Pharoah, Deborah Thompson, et al. Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature*, 447(7148):1087–1093, June 2007.

[169] Christophe Bounaix Morand du Puch, Ewa Barbier, Alexandra Kraut, Yohann Couté, Julia Fuchs, et al. TOX4 and its binding partners recognize DNA adducts generated by platinum anticancer drugs. *Archives of biochemistry and biophysics*, 507(2):296–303, March 2011.

[170] Mehdi Morchikh, Monica Naughtin, Francesca Di Nunzio, Johan Xavier, Pierre Charneau, et al. TOX4 and NOVA1 proteins are partners of the LEDGF PWWP domain and affect HIV-1 replication. *PLoS ONE*, 8(11):e81217, 2013.

[171] Takashi Kajitani, Tetsuya Mizutani, Kazuya Yamada, Takashi Yazawa, Toshio Sekiguchi, et al. Cloning and characterization of granulosa cell high-mobility group (HMG)-box protein-1, a novel HMG-box transcriptional regulator strongly expressed in rat ovarian granulosa cells. *Endocrinology*, 145(5):2307–2318, May 2004.

[172] James W Collins, Kristie M Keeney, Valerie F Crepin, Vijay A K Rathinam, Katherine A Fitzgerald, et al. Citrobacter rodentium: infection, inflammation and the microbiota. *Nature Publishing Group*, 12(9):612–623, September 2014.

[173] Ekaterina P Koroleva, Sydney Halperin, Ekaterina O Gubernatorova, Elise Macho-Fernandez, Cody M Spencer, et al. Citrobacter rodentium-induced colitis: A robust model to study mucosal immune responses in the gut. *Journal of Immunological Methods*, 421:61–72, June 2015.

[174] S Huber, N Gagliani, L A Zenewicz, F J Huber, and L Bosurgi. IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine. *Nature*, 2012.

[175] Li-En Jao, Susan R Wente, and Wenbiao Chen. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proceedings of the National Academy of Sciences of the United States of America*, 110(34):13904–13909, 2013.

[176] Jonathan Perk, Antonio Iavarone, and Robert Benezra. Id family of helix-loop-helix proteins in cancer. *Nature reviews. Cancer*, 5(8):603–614, August 2005.

[177] Cornelis Murre. Helix-loop-helix proteins and lymphocyte development. *Nature Immunology*, 6(11):1079–1086, November 2005.

[178] B L Kee, R R Rivera, and C Murre. Id3 inhibits B lymphocyte progenitor growth and survival in response to TGF-beta. *Nature Immunology*, 2(3):242–247, March 2001.

[179] Ellen V Rothenberg, Ameya Champhekar, Sagar Damle, Marissa Morales Del Real, Hao Yuan Kueh, et al. Transcriptional establishment of cell-type identity: dynamics and causal mechanisms of T-cell lineage commitment. *Cold Spring Harbor symposia on quantitative biology*, 78:31–41, 2013.

[180] Jinfang Zhu, Dragana Jankovic, Alex Grinberg, Liying Guo, and William E Paul. Gfi-1 plays an important role in IL-2-mediated Th2 cell expansion. *Proceedings of the National Academy of Sciences of the United States of America*, 103(48):18214–18219, November 2006.

[181] Jinfang Zhu, Todd S Davidson, Gang Wei, Dragana Jankovic, Kairong Cui, et al. Down-regulation of Gfi-1 expression by TGF-beta is important for differentiation of Th17 and CD103+ inducible regulatory T cells. *Journal of Experimental Medicine*, 206(2):329–341, February 2009.

[182] Ryoji Yagi, Ilkka S Junttila, Gang Wei, Joseph F Urban, Keji Zhao, et al. The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-gamma. *Immunity*, 32(4):507–517, April 2010.

[183] Michael G Constantinides and Albert Bendelac. Transcriptional regulation of the NKT cell lineage. *Current Opinion in Immunology*, 25(2):161–167, April 2013.

[184] Michael G Constantinides, Damien Picard, Adam K Savage, and Albert Bendelac. A naive-like population of human CD1d-restricted T cells expressing intermediate levels of promyelocytic leukemia zinc finger. *The Journal of Immunology*, 187(1):309–315, July 2011.

[185] David Allman, Arivazhagan Sambandam, Sungjune Kim, Juli P Miller, Antonio Pagan, et al. Thymopoiesis independent of common lymphoid progenitors. *Nature Immunology*, 4(2):168–174, February 2003.

[186] Renata Mazzucchelli and Scott K Durum. Interleukin-7 receptor expression: intelligent design. *Nature reviews. Immunology*, 7(2):144–154, February 2007.

[187] Birte Niebuhr, Neele Kriebitzsch, Meike Fischer, Kira Behrens, Thomas Günther, et al. Runx1 is essential at two stages of early murine B-cell development. *Blood*, 122(3):413–423, July 2013.

[188] Takeshi Egawa, Robert E Tillman, Yoshinori Naoe, Ichiro Taniuchi, and Dan R Littman. The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *The Journal of experimental medicine*, 204(8):1945–1957, August 2007.

[189] Vanja Lazarevic, Xi Chen, Jae-Hyuck Shim, Eun-Sook Hwang, Eunjung Jang, et al. T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORγt. *Nature Immunology*, 12(1):96–104, January 2011.

[190] Fuping Zhang, Guangxun Meng, and Warren Strober. Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nature Immunology*, 9(11):1297–1306, November 2008.

[191] Okiru Komine, Keitaro Hayashi, Waka Natsume, Toshio Watanabe, Youichi Seki, et al. The Runx1 transcription factor inhibits the differentiation of naive CD4+ T cells into the Th2 lineage by repressing GATA3 expression. *The Journal of experimental medicine*, 198(1):51–61, July 2003.

[192] Makoto Kuwahara, Masakatsu Yamashita, Kenta Shinoda, Soichi Tofukuji, Atsushi Onodera, et al. The transcription factor Sox4 is a downstream target of signaling by the cytokine TGF-β and suppresses T(H)2 differentiation. *Nature Immunology*, 13(8):778–786, August 2012.