**T-bet and ROR α control lymph node formation
by regulating embryonic innate lymphoid cell differentiation**

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Zusammenfassung

Angeborene lymphoide Zellen (ILCs, engl. für Innate Lymphoid Cells) bilden eine kürzlich beschriebene Familie von Effektorzellen des angeborenen Immunsystems, die in verschiedenste immunologische Prozesse involviert sind. ILCs fehlen somatisch rekombinierte Antigenrezeptoren und können aufgrund ihrer Master-Transkriptionsfaktoren und Effektorfunktionen in drei Hauptgruppen eingeteilt werden. In der Embryonalentwicklung spielt ein spezifisches Mitglied der Typ 3 ILCs, sogenannte LTi (engl. für Lymphoid Tissue inducer) Zellen, eine zentrale Rolle in der Entwicklung von Lymphknoten. Wie die gesamte ILC3 Familie, sind auch LTi Zellen abhängig von dem Master-Transkriptionsfaktor $ROR\gamma t$, was sich in $ROR\gamma t$ -defizienten Mäusen nicht nur durch die Abwesenheit aller ILC3, sondern makroskopisch auch durch fehlende Lymphknoten äußert. ILC3 persistieren bis ins Erwachsenenalter und befinden sich hauptsächlich im Darmgewebe und den assoziierten mesenterialen Lymphknoten, wo sie die Homöostase der Barrierefunktionen, Immunüberwachung, sowie die Typ-3-Gewebeimmunität unterstützen.

Während postnatale Ko-expression der Transkriptionsfaktoren T-bet und $ROR\gamma t$ in spezifischen ILC3-Subpopulationen und deren Bedeutung für Differenzierung, Phänotyp und Funktionen fest etabliert sind, ist der Einfluss von T-bet in fötalen ILC3 und auf die Generation von Lymphknoten noch unbekannt.

Um diese Mechanismen genau zu untersuchen, wurden fötale ILCs mittels Einzelzell-RNA-Sequenzierung detailliert charakterisiert, wodurch eine unerwartete Heterogenität innerhalb der ILC3 mit T-bet-exprimierenden Zellen aufgedeckt wurde. Außerdem wurden $PLZF^+$ ILC-Vorläufer (ILCP, engl. für Innate Lymphoid Cell Precursor) im sich entwickelnden Darm nachgewiesen, was darauf hindeutet, dass der embryonale Darm eine aktive Differenzierungsnische für ILCs während der frühen Entwicklung darstellt.

Weiterhin, bestätigen verschiedene Mausmodelle eine Schlüsselrolle für T-bet in der Regulation der ILC-Differenzierung und der Entstehung von Lymphknoten. Im Detail konnte gezeigt werden, dass die zusätzliche genetische Ablation von T-bet in $ROR\gamma t$ -defizienten Mäusen Differenzierungsentscheidungen in fötalen ILCP zentral beeinflusst. Die Abwesenheit von T-bet in ILCP ermöglichte die Akkumulation von ILCP mit LTi-Aktivität, wodurch die Organogenese von Lymphknoten, unabhängig von $ROR\gamma t$ wiederhergestellt wurde. $PLZF^+$ ILCP von $ROR\gamma t$ /T-bet-Doppeldefizienten Mäusen bestanden bis ins Erwachsenenalter, wo diese Zellen die Darmbarrierefunktionen durch Produktion von IL-22 wiederherstellten. Darüber hinaus erwies sich $ROR\alpha$ als entscheidend für die Entwicklung von $PLZF^+$ ILCP und die damit verbundene Bildung von Lymphknoten. Insgesamt enthüllen diese Ergebnisse eine neue Rolle für T-bet und $ROR\alpha$ in der embryonalen ILC-Differenzierung und der Lymphknoten-Organogenese, und decken die antagonistische Funktion von $ROR\gamma t$ innerhalb der differenzierenden ILCP auf, T-bet entgegenzuwirken.

Summary

Innate lymphoid cells (ILCs) represent a recently described family of innate effector cells lacking rearranged antigen receptors that are implicated in various immunological processes. ILCs are classified into three main groups based on their lineage-specifying transcription factors (TF) and effector functions. During embryonic development, the formation of lymphoid organs critically relies on a specific member of group 3 innate lymphoid cells (ILC3), expressing the master transcription factor retinoic acid related orphan receptor (ROR) γ t and exhibiting lymphoid tissue inducer (LTi) functions. Accordingly, ROR γ t-deficient mice lack ILC3 and do not generate lymph nodes (LN). ILC3 persist into adulthood and mainly reside within the intestinal tissue and associated mesenteric LN, supporting barrier functions in terms of homeostasis and immunosurveillance as well as type 3 tissue immunity.

While it is established that T-box TF T-bet is co-expressed with ROR γ t in a subset of ILC3 emerging postnatally and influencing their differentiation, phenotype and functions, the effect of T-bet on fetal ILC3 biology and its impact on LN generation remains completely unknown.

In order to study the role of T-bet in fetal ILC3 differentiation and functions as well as in LN formation, single-cell RNA sequencing and flow cytometry were applied to characterize fetal ILC subsets revealing an unanticipated heterogeneity within embryonic ILC3 and identifying T-bet⁺ ILC3 subsets within the fetal intestine and mesenteric LN anlage for the first time.

Furthermore, promyelocytic leukaemia zinc finger protein (PLZF)⁺ ILC progenitors (ILCP) were exposed in the developing mouse intestine suggesting the embryonic gut as an active differentiation niche for ILCs during early development.

Importantly, using multiple mouse models, a key role for T-bet in regulating ILC differentiation and LN formation was discovered. Specifically, additional deficiency of T-bet in ROR γ t-deficient mice skewed lineage fate decisions in differentiating fetal ILCP and allowed accumulation of ILCP with LTi activity, thereby rescuing LN organogenesis in a ROR γ t-independent fashion. PLZF⁺ ILCP of ROR γ t/T-bet double deficient mice persisted into adulthood where these cells restored intestinal barrier functions through reinstalled IL-22 production. Moreover, ROR α was found to be critical for the development of PLZF⁺ ILCP and associated LN formation.

Altogether, these data unveil a novel role for T-bet and ROR α in embryonic ILC differentiation and LN organogenesis and further highlight the importance of ROR γ t in counteracting suppressive effects of T-bet within differentiating ILCP.

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1 Introduction

The human immune system is composed of diverse hematopoietic cell types, including lymphocytes from the adaptive as well as from the innate immune system. The family of innate lymphocytes has been expanded recently by the identification of Innate Lymphoid Cells (ILCs), which over the past years emerged as important players not only in the context of infection, cancer and chronic or metabolic diseases, but also in regulation of tissue homeostasis. Intense investigation has contributed to greater understanding of their development, functions and transcriptional regulations in all these processes and further studies will reveal their unique functions as tissue-embedded lymphocytes.

1.1 The biology of innate lymphoid cells

Innate lymphoid cells (ILCs) are characterized by the lack of adaptive antigen receptors and hence represent a distinct arm of the innate immune system. In terms of transcriptional regulation and functions, ILCs can be classified into three major groups that mirror T helper (Th) cell subsets and are termed helper-like ILCs accordingly (**Figure 1**). Group 1 ILCs (ILC1) express IFN γ and depend on T-box transcription factor (TF) T-bet, Group 2 ILCs (ILC2) produce IL-4, IL-5, IL-9, IL-13, AREG and express high levels of GATA-3, while Group 3 ILCs (ILC3) are characterized by production of IL-22, IL-17, lymphotoxin and retinoic acid receptor-related orphan receptor (ROR) γ t expression (Eberl et al., 2014). The innate counterpart of cytotoxic CD8⁺ T cells are represented by natural killer (NK) cells, which, like ILC1, produce IFN γ and depend on T-bet but furthermore express Eomes and high levels of perforin and granzymes demonstrating their superior cytotoxic capacity to degranulate and to kill aberrant cells (Orange et al., 1995; Spits et al., 2016; Vosshenrich et al., 2006). Based on these effector functions, NK cells and ILC1 contribute to type 1 immunity by reacting to intracellular pathogens, such as viruses, certain bacteria or parasites and are fundamental in anti-tumor immunity. ILC2 on the other hand respond to large extracellular parasites and allergens leading to type 2 immunity (Fallon et al., 2006; Monticelli et al., 2011; Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010), while ILC3 combat extracellular microbes like bacteria and fungi conferring type 3 immunity (**Figure 1**) (Buonocore et al., 2010; Cupedo et al., 2009; Lee et al., 2011; Satoh-Takayama et al., 2008; Sonnenberg et al., 2011). However, unlike cells of the adaptive immune system, ILCs seed various tissues already during fetal development and remain tissue-resident throughout lifetime. Strongly enriched at barrier surfaces such as intestine, lung or skin, ILCs emerged as important players in tissue homeostasis with the ability to rapidly react to environmental changes or tissue perturbations. In this context, in the absence of antigen specificity, ILCs are capable of sensing local chemokine or cytokine milieus, recognize damage-

associated as well as pathogen-associated molecular patterns (DAMPs and PAMPs) or crosstalk with other (non-) hematopoietic cells to control diverse physiological processes or orchestrate various immune responses (Sonnenberg and Artis, 2015). ILCs act early on and are an important source of cytokines during the initial phases of a multilayered immune response and hence, together with cells of the adaptive immune system, protect organisms not only against infections but also carefully surveil barrier surfaces under homeostatic conditions. Conversely, the dysregulation of ILCs can result in immune pathology and chronic disorders such as or allergies and related asthma.

Figure 1. The family of innate lymphoid cells. The diversity of ILCs are mirrored by their T cell counterparts from the adaptive immune system. While adaptive lymphocytes are also found in the circulation, ILCs mainly reside at barrier surfaces where they orchestrate multiple host-protective effector functions. Both natural killer (NK) cells and ILC1 produce interferon- γ (IFN γ) amongst other effector molecules to confer type 1 immunity by reacting to intracellular pathogens, such as viruses or intracellular bacteria, and to tumors. Production of type 2 cytokines, including interleukin (IL)-4, IL-5, IL-9, IL-13 or amphiregulin (AREG) promote immunity to helminths and can furthermore be involved in allergic reactions. ILC3 produce IL-17, IL-22 and lymphotoxin to regulate type 3 immunity and control extracellular bacterial infections.

1.2 Development of ILCs

The cellular substrate for the formation of all cells from the hematopoietic lineage are multipotent hematopoietic stem cells (HSCs). Hematopoiesis from HSCs is a continuous process marked by the upregulation of several TFs and a progressive loss of cell-fate potentials. Priming towards

lymphopoiesis and concomitant repression of the myeloid program involves, amongst other TFs, expression of tyrosine kinase FLT3, E2A (also known as TCF3), zinc finger TFs Ikaros and GFI1 (Yoshida et al., 2006). E protein TFs are central components in this step as these promote specification and commitment to adaptive T and B cell fates. The lymphoid-committed resulting cell population is named the common lymphoid progenitor (CLP) and has a restricted potential to generate T and B lymphocytes but also ILCs. Under the influence of IL-2R γ c signaling, in the main provided by IL-7, further differentiation towards the ILC lineage is promoted by the inhibitor of DNA-binding 2 (ID2), an ID family member of transcriptional repressors. Specifically, ID2 antagonizes DNA binding activities of E proteins thereby inhibiting T and B cell potential while strongly promoting ILC generation (Boos et al., 2007; Harly et al., 2018; Klose et al., 2014; Yokota et al., 1999). Activation of ID2 is concomitantly promoted by the TF nuclear factor IL-3 NFIL3 (also known as E4BP4) identifying these factors as critical controllers of innate lymphocyte differentiation (Geiger et al., 2014; Seillet et al., 2014a; Xu et al., 2015). Subsequent additional key transcriptional circuitries by a set of genes including TOX, TCF1 and GATA3 dedicates to the innate lymphoid cell progenitor (ILCP) as shown by strong developmental defects in mice gene-deficient for one of these TFs (**Figure 2**) (Aliahmad et al., 2010; Mielke et al., 2013; Seehus et al., 2015; Yagi et al., 2014; Yang et al., 2015). Moreover, several other TFs such as ETS1, ROR α or PLZF have been shown to be expressed by committed ILCPs (Constantinides et al., 2014; Halim et al., 2012; Walker et al., 2019; Wong et al., 2012; Yu et al., 2016; Zook et al., 2016). Succeeding differentiation of ILCPs towards diversification of main ILC groups finally manifests by the expression of lineage-specifying TFs, resulting in emergence of mature ILC subsets with distinct functional properties described above.

Figure 2. Model of ILC development. The developmental program of ILCs is characterized by the sequential expression of transcription factors and successive loss of differentiation potential and is mainly based on studies in mice. The common lymphoid progenitor (CLP) originates from hematopoietic stem cells (HSC) and gives rise to not only B and T cells but also to innate lymphoid cells. Upregulation of ID2 (inhibitor of DNA-binding 2) is crucial for suppression of adaptive cell fates and progression towards the innate lymphoid cell progenitor (ILCP). Subsequent expression of the master transcription factors Eomes (Eomesodermin), T-bet (T-box transcription factors), GATA3 (GATA binding protein 3) and ROR γ t (RAR-related orphan receptor γ t) define the diverse mature ILC lineages. The different progenitor populations have been found in various locations indicated in the yellow box with early progenitors mainly being detected in the circulation or in niches closely linked to it while the ILCP has been detected in several tissues like tonsil or lung. Mature lineages mainly reside within tissues or at mucosal surfaces such as intestine or lung. FLT3, receptor-type tyrosine-protein kinase FLT3; E2A, E2A immunoglobulin enhancer-binding factors E12/E47; GFI1, Growth Factor Independence 1; RAG1/2, Recombination Activating Gene 1/2; TCF1, T Cell Factor 1; EBF-1, Early B-Cell Factor 1; ID2, Inhibitor of DNA-binding 2; NFIL3, Nuclear Factor, Interleukin 3 Regulated; ETS1, avian erythroblastosis virus E26 homolog-1; TOX, thymocyte selection-associated high mobility group box protein; ROR α , RAR-related orphan receptor α ; PLZF, (promyelocytic leukemia zinc finger; IL-7, interleukin-7.

In embryos, HSCs originate from the yolk sac-derived hemogenic endothelium around embryonic day E10 in mice. As development progresses, CLPs are found in fetal liver (FL) and peripheral LN (Simic et al., 2020) as sites of lymphopoiesis and eventually the BM represents the main niche for hematopoiesis later during ontogeny. Studies in mice and human have identified circulating ILCP (Constantinides et al., 2014; Harly et al., 2018; Klose et al., 2014; Lim et al., 2017; Nagasawa et al., 2019; Renoux et al., 2015; Xu et al., 2019), as well as tissue-resident progenitor pools detected in the fetal mouse intestine (Bando et al., 2015) and adult mouse lung (Zeis et al., 2020) or human tonsil (Montaldo et al., 2014; Renoux et al., 2015; Scoville et al., 2016) suggesting that ILC differentiation may occur at sites other than FL or BM (Figure 2).

1.3 The ILC3 family

Among all lymphocytes, type 3 innate lymphoid cells are the first lymphocytes to appear during embryonic development to organize the formation of lymphoid structures. These cells furthermore confer the first line of defence in newborns until lymphocytes of the adaptive immune system are functionally generated. Later in life, ILC3 are found to be highly enriched in the mucosa of the intestine where they play an essential role in regulating intestinal homeostasis and tissue health but also type 3 immunity. Compared to ILC1 and ILC2, ILC3 represent the most heterogeneous ILC family in adult individuals and can be further divided into three major subpopulations, which are described in the following section.

1.3.1 ILC3 heterogeneity

Subsets of ILC3

In adult mice, ILC3 subsets include natural cytotoxicity receptor (NCR) NKp46⁺ and NKp46⁻ ILC3s (Klose et al., 2013; Sawa et al., 2010) and also lymphoid tissue inducer (LTi) cells. The latter, named after their function to facilitate formation of lymphoid organs during embryonic development, arise in FL and can be characterized by expression of their master regulator ROR γ t and a subset additionally by CD4 on their surface (**Table 1**) (Cupedo et al., 2004; Eberl et al., 2004; Mebius et al., 2001). LTi cells accumulate in the embryonic LN anlagen and initiate LN organogenesis through surface presentation of lymphotoxin (LT), specifically the heterotrimer LT $\alpha_1\beta_2$, that signals to specialized stromal cells. Mice deficient for ROR γ t fail to develop LTi cells and consequently lack all LN and Peyer's patches (PP) (Eberl et al., 2004; Sun et al., 2000). After birth, a population of CCR6⁺CD4^{+/-} ILC3, phenotypically resembling LTi cells, is present in spleen, intestine, and LN along with NKp46⁺ and NKp46⁻ ILC3 subsets (**Table 1, Figure 3**) (Cupedo et al., 2009). However, LTi cells from the adult stage are unable to facilitate organogenesis of secondary lymphoid organs (Kim et al., 2007; Mariathasan et al., 1995) and rather play a role in regulating the quality and magnitude of adaptive immune responses (Von Burg et al., 2014; Hepworth et al., 2013; Kruglov et al., 2013). The relationship between embryonic LTi cells and those found in adults remains unclarified to date. A model suggests that different waves of ILC3 generation facilitates the generation of functionally different ILC3 subsets, a term called layered ontogeny (McGrath et al., 2015a; Simic et al., 2020). After birth, subsets of non-LTi, described as NCR^{+/-} ILC3, are present and populate gastrointestinal tissues such as small and large intestine as well as mLN and can furthermore be found in spleen. Importantly, NKp46⁺ ILC3 emerge together with the colonization of the commensal microflora and indispensably express T-bet in addition to ROR γ t (Sanos et al., 2009; Satoh-Takayama et al., 2008; Sawa et al., 2010).

In humans, LTi cells could be detected within the fetal mesentery and spleen isolated from the first trimester at 8-9 weeks of gestation; however, CCR6 cannot be used as an identifying marker

for LT_i/LT_i-like cells since all ROR γ ⁺ ILC3 are CCR6⁺ (Cupedo et al., 2009; Shikhagaie et al., 2017). Instead, NRP-1 has been used herein which has been also shown to be expressed by mouse LT_i cells (Robinette et al., 2015; Shikhagaie et al., 2017). Of note, various single-cell RNA sequencing approaches of cells isolated from tissues adult individuals (blood, tonsil, spleen, lung, colon) though have failed to identify a LT_i signature as a separate subcluster from the rest of ILC3 (Bar-Ephraim et al., 2017; Björklund et al., 2016; Mazzurana et al., 2021; Yudanin et al., 2019). Postnatally, human ILC3 subsets are found in tonsils, intestine, skin, LN, spleen, nasal polyps and lung can be distinguished based on CD56 and NKp44 expression, of which the NKp44⁺ subset resembles LT_i cells (Cella et al., 2009; Glatzer et al., 2013; Hoorweg et al., 2012). Notably, NKp44⁺ ILC3 largely co-express NKp46; but, contradictory to mouse NK46⁺ ILC3, human ILC3 do not express T-bet *ex vivo*.

Taken together, there exists a remarkable diversity among ROR γ ⁺ ILC3 postnatally, which can be characterized by a set of surface markers and by co-expression of T-bet in mouse (**Table 1**).

Table 1. Murine ILC3 markers.

	LT _i	NCR ⁻ ILC3	NCR ⁺ ILC3
Markers			
CD127	+	+	+
IL-23R	+	+	+
IL-1R1	+	+	+
CCR6	+	-	-
CD4	+/-	-	-
CXCR5	+	-	-
NRP-1	+	-	-
NK1.1	-	-	lo/-
NKp46	-	-	+
DNAM1	-	-	+
c-Kit	+	lo	lo
CD49a	-	+/-	+
CD200R1	-	+	+
Transcription factors			
ROR γ ^t	+	+	+/lo
T-bet	-	+/-	+
GATA3	lo	lo	lo
Cytokines			
IFN γ	-	-/lo	-/lo
IL-22	+	+	+
IL-17	+	+/-	-
LT	+	+/-	-

+ indicates high expression, - indicates no expression, +/- indicates bimodal expression, lo indicates low expression; LT, lymphotoxin
Applicable according to published reports (Klose et al., 2013; Robinette et al., 2015; Verrier et al., 2016)

Antagonizing signals within ILC3 lineages

In light of the existence of several ILC3 subsets, particularly factors regulating subset classifications were identified. For example, it was proposed that the upregulation of NKp46 and T-bet involves, besides IL-23, Notch signaling and the microbiota (Klose et al., 2013, 2014; Rankin et al., 2013; Sawa et al., 2010; Sciumè et al., 2012). Furthermore, a sustained Notch pathway is required for the maintenance of NKp46⁺ ILC3 while the cytokine transforming growth factor- β (TGF- β) exerts opposing signals inhibiting their generation (Chea et al., 2016a; Viant et al., 2016). With the acquisition of T-bet, NKp46⁺ ILC3 become sensitive to IL-12 and display an “NK-ness” program while transcriptional profiles within NCR⁺CD4^{+/−} ILC3 subsets are almost identical, as shown using bulk RNA sequencing (Rankin et al., 2016; Robinette et al., 2015). Thus, T-bet seems to regulate ILC3 identity not only phenotypically but also transcriptionally and tunable co-expression of T-bet and ROR γ t serves as a molecular switch controlling gene expression patterns.

Data obtained in T cells point towards a regulatory role of T-bet in the development of the Th1-like Th17 lineage (Lazarevic et al., 2011). Mechanistically, T-bet indirectly inhibits ROR γ t and consequently represses Th17 lineage. Regulations between ROR γ t- and T-bet-driven programs might potentially also exist in ILC3 co-expressing the two TFs, as shown in T-bet-deficient mice, in which NKp46[−] ILC3 display enhanced expression of ROR γ t and are increased in numbers (Schroeder et al., 2020). Interestingly, a role for ROR γ t in counteracting T-bet functions in ILC3 has been proposed, based on the observation that in heterozygous *Rorc(gt)^{GFP/wt}* mice, the lack of one copy of the gene encoding ROR γ t, results in a shift in ILC3 subset equilibrium towards NKp46-expressing ILC3 (Walker et al., 2019; Zhong et al., 2016). However, the detailed mechanisms of ROR γ t and T-bet regulation within ILC3, in particular prenatally, remain unclarified to date.

Figure 3. Antagonizing factors regulate ILC3 subsets during ontogeny. Lymphoid tissue inducer (LTi) cells are among the first lymphocytes to develop during ontogeny. These cells uniformly express RORγt and are recruited to LN anlagen where they mature further to produce lymphotoxin enabling formation of lymph nodes. While RORγt⁺ LTi cells represent the majority of ILC3 prenatally, NKp46⁺ ILC3 co-expressing T-bet emerge postnatally with colonization of commensal bacteria, Notch signaling and IL-23. Within NCR⁺ ILC3 RORγt and T-bet seem to counteract each other's transcriptional programs. Postnatal ILC3 subsets are involved in IL-22 dependent maintenance of barrier function and type 3 immunity. How LTi cells relate to NCR⁻ ILC3 and which signals could drive their interconversion is unclarified to date.

1.3.2 ILC3 plasticity

Although generally transcriptionally similar, bulk sequencing of small intestinal NKp46⁺ ILC3 and LTi cells demonstrated distinct transcriptional states within these and single-cell sequencing analyses of small intestinal ILCs furthermore discriminated between five discrete ILC3 transcriptional states (Gury-BenAri et al., 2016; Robinette et al., 2015). This suggests that there might exist dynamic transcriptional and functional states within ILC3, as the broad range of environmental stimuli and/or cellular interactions present in the intestine might require ILC3 to adjust to these conditions and might reflect their need to regulate their identity. As mentioned in section 1.3.1, besides commonly designated NCR⁺ ILC3 and LTi cells, a population of NKp46⁻ CCR6⁻RORγt⁺ ILC3 subsets, termed “double-negative” (DN) ILC3, exists. DN ILC3 have been described to represent a pool of precursors cells that are able to upregulate T-bet and acquire NKp46, a process driven by IL-23, Notch signaling and microbiota (Klose et al., 2013; Rankin et al., 2013; Verrier et al., 2016; Vonarbourg et al., 2010). Reversely, *Ncr1* (encoding NKp46) fate-mapping studies revealed ILC3 that had switched to a DN ILC3 phenotype upon transforming growth factor-β (TGF-β) signals (Verrier et al., 2016; Viant et al., 2016). Interestingly, although

currently no data on the plasticity within LT_i cells have been demonstrated in the literature, some CCR6⁺CD4⁺ LT_i cells also exhibited a history of NKp46 expression in a study suggestive of plastic changes within the LT_i compartment (Verrier et al., 2016).

In addition to the NCR⁺ and LT_i ILC3 subtypes, an ILC3-ILC1 transitional subset has been described in adult mice and men that resembles ILC1 and is referred to as ex ILC3. ROR γ ⁺T-bet⁺ ex ILC3 have been shown to differentiate from NKp46⁺ ILC3 in an IL-12 environment downregulating ROR γ and only expressing T-bet as revealed in fate-mapping studies (Figure 4) (Bernink et al., 2015; Klose et al., 2013, 2014; Vonarbourg et al., 2010). Thus, interconversion of NKp46⁺ ILC3 to ex-ILC3 producing IFN γ is determined by a local cytokine milieu and associated to pathology in mouse models of colitis or disease in Crohn's patients (Bernink et al., 2013; Cella et al., 2019; Verrier et al., 2016; Viant et al., 2016; Vonarbourg et al., 2010). Gene expression analyses suggest that NKp46⁺ROR γ ^{lo} ILC3 might reflect a cell population converting into ex ILC3 (Figure 4) (Robinette et al., 2015). In this context, the TF c-Maf has been reported to balance ILC3/1 subsets by antagonizing T-bet-driven type 1 programs and by enforcing ILC3 identity through support of ROR γ activity and type 3 effector transcriptomes (Parker et al., 2020; Pokrovskii et al., 2019; Tizian et al., 2020). Importantly, human studies have shown that this plastic phenotype is reversible and possibly induced by homeostatic levels of cytokines, highlighting that a blockade of inflammation-polarizing signals could serve as a therapeutic option to resolve inflammation and pathology (Bernink et al., 2013, 2015; Viant et al., 2016). Overall, this phenotypic instability suggests a major role for environmental signals and continual challenges in tuning interconversion of ILC3 subsets along with functional plasticity, and highlights the challenge to categorize the various ILC3 populations based on a very limited number of surface markers.

Figure 4. Environmental signals and the interplay of transcription factors ROR γ and T-bet regulate ILC3 plasticity.

Representative flow cytometry plot of ILCs isolated from the small intestine of adult *Rorc(gt)^{GFP/wt}* reporter mice. Reporter signal ROR γ -GFP defines ILC3 where NKp46⁺ subsets consist of lymphoid tissue inducer (LT_i) cells and natural cytotoxicity receptor (NCR)⁺ ILC3. Microbiota, Notch and Ahr signaling are involved in upregulation of T-bet and consequent differentiation of NCR⁺ ILC3 expressing NKp46. Through IL-12 signaling this subset is able to turn off ROR γ expression and convert to ex ILC3. In order to differentiate between ILC1 and ex ILC3 ROR γ -fate map mice should be used. To delineate NK cells from ILC1 transcription factors T-bet and Eomes should be analyzed. RA, retinoic acid; Ahr, aryl hydrocarbon receptor.

1.3.3 Functions of ILC3 prenatally: lymph node organogenesis

Before birth, ROR γ t⁺ ILC3 are considered a rather homogeneous population mainly comprising of LT_i cells, which can be detected as early as murine embryonic day E12.5 (12.5 days after conception) in FL (Mebius et al., 1997; Veiga-Fernandes et al., 2007). They can be classified based on the expression of CD4 into LT_{i0} and LT_{i4} cells (Sawa et al., 2010; Vonarbourg et al., 2010) and guide a highly programmed series of events that includes the interaction between stromal cells and LT_i cells, eventually leading to the generation of lymph nodes in the fetus (Boos et al., 2007; Mebius, 2003). In mice, LT_i occurrence and initiation of lymphoid organs occurs between E12.5 until E15.5 at specific locations within the embryo. Specifically, LT_i cells egress out from the FL and enter the sites of future lymphoid organ development through expression of integrin $\alpha_4\beta_7$ on LT_i cells that enables rolling and firm adhesion on its ligand MadCam-1 expressed by high endothelial venules (Mebius et al., 1996). LT_i cells are further attracted by chemokines such as CXCL13 produced by stromal cells while IL-7 additionally stimulates their accumulation and local maturation. Interestingly, expression of CXCL13 (the chemokine binding to CXCR5) in stromal organizer cells as well as ROR γ t in LT_i cells is induced by retinoic acid (RA) signaling *in utero* (Van De Pavert et al., 2009, 2014). Thus, LT_i cell recruitment and maturation is regulated by the mother's food consumption, which eventually controls the size of secondary lymphoid organs and the efficiency of immune responses in adult offspring (Van De Pavert et al., 2014). At place, LT_i cells provide the heterotrimer LT $\alpha_1\beta_2$ (Mebius et al., 1997) to induce activation of stromal organizer cells to differentiate towards specialized lymphoid tissue organizer (LT_o) cells (De Togni et al., 2014). Receptor activator of NF- κ B (RANK)-RANKL (Ligand) interactions between LT_i and LT_o cells further orchestrate lymph node development by stimulating LT $\alpha_1\beta_2$ expression and LT_i cell accumulation (Onder et al., 2017). Although molecular mechanisms of interactions between LT_i and LT_o cells have been a subject of studies over several years, the detailed sequence of events that generate individual specific LN has remained unclear. In this context, it appears that in different mouse knockout models specific LN are present or absent suggesting that distinct processes operate during particular LN generation (Table 2). For example, the developmental pathways for mLN and PP differ in their requirements for chemokines/growth factors (Coles et al., 2010) (see next section 1.3.4 below).

Taken together, LT_i cells are central in inducing the signals for lymph node development and several adhesion molecules and chemokines are needed for their attraction, retention and activation within the developing lymph node during embryogenesis (Figure 5).

Figure 5. Model for development of secondary lymphoid organs. (1) Induction of organizing centers. Retinoic acid (RA), possibly produced by nerve fibers, induces expression of CXCL13 by mesenchymal cells to attract LTi cells from blood. Local IL-7- and RA-signaling in LTi cells leads to their maturation and expression of the heterotrimer lymphotoxin (LT) $\alpha_1\beta_2$ on their surface. **(2) Clustering of LTi and LTo cells.** LT β signaling provided to stromal cells leads to their differentiation into lymphoid tissue organizer (LTo) cells. Further interactions via VCAM1- $\alpha_4\beta_7$ and RANK-RANKL facilitates retention and accumulation of LTi cells. **(3) Architectural maturation and entry of lymphocytes.** Eventually, a large cell cluster forms and blood vessels start to develop into high endothelial venules, which allows cells to enter from the bloodstream.

1.3.4 Functions of ILC3 prenatally: Peyer's patch development

PP are organized lymphoid follicles located within the mucosa layer of the small intestine, which harbor not only T and B cells but also dendritic cells (DCs), macrophages and neutrophils, thus facilitating crosstalk between these immune cells. Microfold (M) cells are located within PP and actively transport antigen into the follicles to initiate immune responses indicating PP as important locations in immune surveillance. In mice, E15.5 marks the beginning of PP development. First, VCAM-1⁺LT β R⁺ stromal cells stochastically locate to the antimesenteric side of the small intestine (Adachi et al., 1997; Patel et al., 2012). Second, between E15.5 and E17.5 CD11c⁺CD127⁺CD4⁺ROR γ t⁺ termed lymphoid tissue initiator (LTi) cells providing RET signaling together with ROR γ t⁺ LTi cells providing LT β signaling to VCAM-1⁺LT β R⁺ stromal cells leads to their maturation into LTo cells and to the induction of PP anlagen (Hashi et al., 2001; Patel et al., 2012; Veiga-Fernandes et al., 2007). LTo cells in turn express chemokines, adhesion molecules and cytokines required for recruitment, retention and survival of LTi cells. In this regard, intact LT β , CXCR5, as well as IL-7R pathways have been shown to be essential for proper PP development (Table 2). From E17.5 on, circulating lymphocytes are attracted to enter the structures and fill up the niches (Adachi et al., 1997). Interestingly, inhibition of RA signaling only reduces LN dimensions but completely abolishes PP development suggestive of different maternal-derived nutrient requirements in the development of these lymphoid structures (Van De Pavert et al., 2014).

Overall, LTi cells form the basic architecture during embryonic development and induce PP structures (variable numbers ranging from 5-12 in mice) before entry of mature lymphocytes after birth. Interestingly, our current understanding revealed differing mechanisms in the development of LN versus PP (**Table 2**).

Table 2. Development of LTi cells, mLN and PP in mutant mice.

	LTi	mLN	PP	References
<i>Id2</i> ^{-/-}	-	-	-	(Yokota et al., 1999)
<i>Rorc(gt)</i> ^{GFP/GFP}	-	-	-	(Eberl et al., 2004)
<i>Il7</i> ^{r/-}	+	+	-	(Cao et al., 1995)
<i>Il7</i> ^{-/-}	+	+	+	(von Freeden-Jeffry et al., 1995)
<i>Ltα</i> ^{-/-}	+	-	-	(Banks et al., 1995)
<i>Ltβ</i> ^{-/-}	+	+	-	(Koni et al., 1997)
<i>Cxcr5</i> ^{-/-}	+	+	0-2	(Ansel et al., 2000)
<i>Cxcl13</i> ^{-/-}	+	-	-	(Förster et al., 1996)
<i>Tnfsf11</i> ^{-/-}	+	-	+	(Kim et al., 2000)
<i>Kit</i> ^W / <i>kit</i> ^W	+	+	++	(Finke et al., 2002)
<i>Tnf</i> ^{-/-}	+	+	reduced	(Pasparakis et al., 1997)

+ indicates presence, - indicates absence

1.3.5 Functions of ILC3 postnatally: tissue homeostasis

IL-22-mediated barrier surveillance

After birth, LTi cell frequency and absolute numbers drop and ILC3 heterogeneity increases simultaneous to establishment of symbiotic microbiota (Sawa et al., 2010). In adult individuals, ILC3 mainly locate to intestinal tissues as well as mLN and spleen with an expected half-life of 3-4 weeks in mice (Sawa et al., 2010). There, both human and mouse ILC3 subsets all tonically produce IL-22 under homeostatic conditions including NCR^{+/-} and LTi-like ILC3 to act as major regulators in protection against commensal microbes (Satoh-Takayama et al., 2008; Sawa et al., 2011). In more detail, IL-22 availability in the intestinal tissue is tuned by the microbiota through IL-1α and IL-23 released from dendritic cells or macrophages, which both stimulate ILC3. IL-22 in turn acts selectively on non-hematopoietic cells like epithelial or stromal cells to induce production of antimicrobial peptides (AMP), such as Reg proteins or β-defensins, thereby controlling bacterial amounts and maintaining epithelial barrier homeostasis. Loss of ILC3-derived IL-22 results in barrier breakdown and translocation of intestinal bacteria to peripheral organs causing systemic inflammation (Sanos et al., 2009; Satoh-Takayama et al., 2008; Sonnenberg et al., 2012). Additionally, IL-22 triggers fucosylation of the intestinal epithelium – an important mechanism to maintain host-microbiota symbiosis, as commensal bacteria adapted

to utilize fucose as a dietary carbohydrate while pathogenic bacteria lack this ability (Goto et al., 2014; JM et al., 2014; Pham et al., 2014). Optimal production of IL-22 by ILC3 has been shown to be modulated by a variety of dietary-derived vitamins, for example vitamin D or also vitamin A-derived RA, the latter similar to LT_i regulation *in utero* (Konya et al., 2018; Van De Pavert et al., 2014; Spencer et al., 2014). Moreover, the TF aryl hydrocarbon receptor (Ahr) can be activated in ILC3 by compounds derived from cruciferous vegetables such as broccoli to regulate IL-22 expression in ILC3 (Kiss et al., 2011; Lee et al., 2011; Qiu et al., 2012, 2013). Overall, ILC3 subsets in the gastrointestinal tract limit the replication and dissemination of commensal bacteria and thus are essential for the maintenance of barrier function and tissue homeostasis in an IL-22-dependent manner (**Figure 6**). Cytokine production can be modulated by multiple factors in response to food intake.

ILC3 regulation of B cell-derived IgA

An additional layer of intestinal homeostasis is provided by IgA-producing B cells that control commensal microbiota size and composition. On the one hand, ILC3 are positioned within PPs where they produce soluble LT α_3 thereby controlling T cell homing to the gut to promote T cell-dependent IgA production by B cells (Kruglov et al., 2013). On the other hand, clustered LT_i cells facilitate development of structures denoted as Cryptopatches (CP) that develop in the first week of life, which will mature into isolated lymphoid follicles (ILFs) as they become populated with B cells producing IgA in response to microbial and dietary signals (Bouskra et al., 2008; Kanamori et al., 1996; Kiss et al., 2011). Formation of ILF from CP requires LT β R signaling in stromal cells provided by clustered LT_i cells presenting membrane bound LT $\alpha_1\beta_2$ on their surface (Kang et al., 2002; Lgering et al., 2010; Tsuji et al., 2008). Taken together, ILC3 directly regulate ILF development and indirectly IgA levels in the gastrointestinal tract via membrane bound LT $\alpha_1\beta_2$ and soluble LT α_3 (**Figure 6**).

ILC3 regulation of commensal bacteria-specific T cells

ILC3-regulate intestinal homeostasis not only by production of IL-22 and LT but also by directly interacting with T cells. In particular, LT_i cells localize in close proximity to T cells in order to modulate and orchestrate their immune responses through multiple pathways. For example, LT_i cells constitutively express major histocompatibility complex class II (MHCII) endowing them the ability to present antigen to CD4⁺ T cells thereby limiting commensal bacteria-specific CD4⁺ T cell responses (**Figure 6**). T cell death is on the one hand induced due to absence of co-stimulatory molecules upon MHCII presentation and secondly by out competition for local IL-2 necessary for T cell proliferation by ILC3 (Von Burg et al., 2014; Hepworth et al., 2013, 2015). Lack of ILC3-intrinsic MHCII in mice results in development of microbiota-dependent T-bet-driven colitis and identify intestinal ILC3 as important players that limit pathological adaptive immune responses to commensal bacteria.

Figure 6. ILC3 in gut homeostasis. Under homeostatic conditions ILC3 are central orchestrators of intestinal tolerance. In particular, ILC3 are a dominant source of interleukin (IL)-22 at steady state. IL-22 acts on epithelial cells to produce antimicrobial peptides such as Reg3 γ and Reg3 β and furthermore induces fucosylation to maintain segregation of commensal bacteria. In addition, ILC3 regulate IgA production of B cells via soluble LT α_3 and limit CD4 $^+$ commensal T cells in a cell-cell contact dependent manner mediated by major histocompatibility complex (MHC)II.

1.3.6 Functions of ILC3 postnatally: protective mucosal immunity

While ILC3 emerged as crucial cells in the maintenance of tissue homeostasis and gut-mucosal tolerance, they can furthermore be involved in inflammation and confer immunity to pathogens. The contribution of ILC3-derived IL-22 in host protective immunity has been assessed in various infection models including *Citrobacter rodentium* (a model organism for clinically relevant enteropathic *Escherichia coli* infections) (Sato-Takayama et al., 2008; Sawa et al., 2011; Sonnenberg et al., 2011), *Enterococcus faecalis* (Pham et al., 2014) and rotavirus (Hernández et al., 2015). Of note, in the presence of the adaptive immune system, especially T cells, ILC3 are dispensable for the control of *C. rodentium* infection; however, ILC3-derived IL-22 is required to control the early phase of infection before antigen-specific T cell responses are raised and thus represent a first line of defense, especially in newborns (Basu et al., 2012; Cella et al., 2009; Klose et al., 2014; Rankin et al., 2016; Song et al., 2015). These data suggest complementary and redundant functions of ILC3 in multilayered immune responses including innate as well as adaptive lymphocytes. In addition to IL-22, ILC3 are also able to secrete IL-17, a cytokine crucial

for resistance to fungal oropharyngeal infections such as with *Candida albicans* (Gladiator et al., 2013; Okada et al., 2015). Remarkably, ILC3-derived IL-17 also showed airway protective responses against *Klebsiella pneumoniae* (Xiong et al., 2016). Furthermore, T-bet co-expressing ILC3 are concomitantly able to produce IFN γ , a cytokine essential to control *Salmonella typhimurium* infection (Goto et al., 2014; Klose et al., 2013).

1.3.7 Dysregulation of ILC3 postnatally: implications in disease

The continuous stimulation of ILC3 triggers functional responses that under normal conditions promote barrier integrity and homeostasis; however, dysregulated IL-22/IL-17-producing ILC3 are associated with multiple chronic human inflammatory diseases such as inflammatory bowel disease (IBD) including Crohn's disease (CD) and Ulcerative colitis (UC) (Geremia et al., 2011). Detrimental ILC3 responses have also been associated in several mouse models of colitis, induced by *Helicobacter hepaticus*, *Helicobacter typhlonius*, *S. typhimurium* or anti-CD40, and related to production of IL-17, GM-CSF and IFN γ (Buonocore et al., 2010; Klose et al., 2013; Pearson et al., 2016; Powell et al., 2012; Vonarbourg et al., 2010). Despite the beneficial effects of IL-22 in homeostasis, unfavorable effects of this cytokine after tissue damage have been reported. In this context, ILC3-derived IL-22 was implicated in development of colorectal cancer (Grivennikov et al., 2012; Hernandez et al., 2018; Kirchberger et al., 2013). Moreover, harmful ILC3 responses have been observed in the skin of patients with psoriasis in which IL-22 revealed as a key driver of epidermal thickening and identified ILC3 to participate in pathology (Teunissen et al., 2014; Villanova et al., 2014). Additional IL-17 production establishes ILC3 as harmful innate lymphocytes in the skin from mouse models of psoriasis-like disease (Pantelyushin et al., 2012). Further, using a mouse model of obesity-induced asthma, IL-17 producing ILC3 expand in response to IL-1 β -producing macrophages to drive airway hyper-responsiveness (Kim et al., 2014).

In summary, inappropriate activation of ILC3 may contribute to chronic (intestinal) inflammation (**Figure 7**) and highlight the importance to understand the circumstances and signals leading to dysregulation and pro- versus anti-inflammatory ILC3 phenotypes in order to prevent or treat human diseases.

Figure 7. ILC3 in inflammation. Under inflammatory conditions, activated ILC3 may induce pathology through excessive production of IL-22 and IL-17 increasing epithelial permeability resulting in failure to maintain intestinal barrier. Furthermore, dysregulated IL-22 and prolonged chronic inflammation inducing ILC3 conversion to ILC1 can result in epithelial cell damage and potentially progress to colorectal cancer.

2 Aims

Whereas mature ILC3 isolated from adult mice have been extensively studied, their development, heterogeneity and plasticity during embryonic development are poorly understood. To date, LT_i cells expressing the master transcription factor ROR γ t are considered a rather homogenous cell population that develops early in fetal life and plays a crucial and non-redundant role in LN formation. However, whether heterogeneous ILC3 subsets displaying differential transcriptomic as well as phenotypic properties establish already before birth remains unclear. While the antagonistic interplay of ROR γ t and T-bet in ILC3 has only been elucidated postnatally, the roles of these two TFs during fetal development, especially in LT_i cells, have not been investigated to date.

Hence, the overall aim of this thesis was firstly to characterize embryonic ILCs with a particular focus on ILC3, in order to define their transcriptional and phenotypic heterogeneity by applying in-depth analyses such as multicolor flow cytometry as well as single-cell RNA sequencing. Secondly, as T-bet-expressing ILC3 subsets emerged during this approach, the effect of T-bet on embryonic ILC3 function, namely LN formation, was assessed through analysis of multiple mouse models. Thirdly, these steps led to the identification of a complex interplay between ROR γ t, T-bet and ROR α , acting already early on as crucial regulators of ILCP differentiation during embryogenesis.

3 Materials and Methods

3.1 Mice

Tbx21-ZsGreen mice (Zhu et al., 2012), *Rorc(gt)^{GFP/wt}* (Eberl et al., 2004), *Rorc(gt)^{Δex4}* (Sun et al., 2000), *Tbx21^{-/-}* (Finotto et al., 2002) and *Rag2^{-/-}* (Shinkai et al., 1992) were generated as described. *Rorc(gt)^{cre}* (Eberl and Litman, 2004) and *Tbx21^{cre}* (Haddad et al., 2013) mice were crossed to *R26R^{eYFP}* and used for *Rorc(gt)*- or *Tbx21*-fate mapping studies. *Rorc(gt)^{cre}* was carried only by female breeders to prevent germline YFP expression. To obtain mice with specific deletion of *Rorc* and/or *Tbx21* in *Il7r*-expressing cells, *Rorc^{fl/fl}* (Choi et al., 2016) and/or *Tbx21^{fl/fl}* (purchased from Jackson, stock ID 022741) and/or *Rora^{fl/fl}* (Oliphant et al., 2014) were crossed to *Il7r^{cre}* (Schlenner et al., 2010) mice in cooperation with David Withers, Birmingham. *Rag2^{-/-} Il2rg^{-/-}* mice were crossed to express the CD45.1 in our facility. Timed pregnancies were accomplished by mating in the evening; evidenced copulation was checked in the morning and females then considered E0.5. All mice were bred under specific pathogen-free conditions in the animal facility of the Federal Institute for Risk Assessment (Berlin, Germany) and the Research Institute for Experimental Medicine (FEM) of the Charité (Berlin, Germany). Animal handling and experiments were conducted according to the German animal protection laws and approved by the responsible governmental authority (Landesamt für Gesundheit und Soziales).

Table 3. Experimental models: mouse strains

Reagent or resource	Source	Identifier
<i>Tbx21-ZsGreen</i>	(Zhu et al., 2012)	N/A
<i>Rorc(gt)^{GFP/wt}</i>	(Eberl et al., 2004)	JAX #007572
<i>Rorc(gt)^{Δex4}</i>	(Sun et al., 2000)	JAX #007571
<i>Tbx21^{-/-}</i>	(Finotto et al., 2002)	JAX #004648
<i>Rag2^{-/-}</i>	(Shinkai et al., 1992)	MGI #1858556
<i>Rorc(gt)^{cre}</i>	(Eberl and Litman, 2004)	JAX #022791
<i>Tbx21^{cre}</i>	(Haddad et al., 2013)	JAX #024507
<i>R26R^{eYFP}</i>	(Srinivas et al., 2001)	JAX #006148
<i>Il7r^{cre}</i>	(Schlenner et al., 2010)	MGI #4441349

<i>Rorc</i> ^{fl/fl}	(Choi et al., 2016)	JAX #008771
<i>Tbx21</i> ^{fl/fl}	N/A	JAX #022741
<i>Rora</i> ^{fl/fl}	(Oliphant et al., 2014)	N/A
<i>Rag2</i> ^{-/-} <i>Il2rg</i> ^{-/-}	N/A	JAX #002479

3.2 Tissue dissociation

Single cell suspensions from intestinal lamina propria of adult animals were obtained as described before (Paclik et al., 2015). In short, intestines were isolated and PPs were removed. Tissue was cleaned, longitudinally opened and chopped into pieces of 2 cm, followed by two rounds à 15 min at 37°C of epithelial cell dissociation using Hank's balanced salt solution without calcium and magnesium (HBSS^{-/-}, Gibco) supplemented with 2% FCS, 10 mM HEPES buffer (Sigma), 1 mM DTT (ThermoFisher) and 5 mM EDTA (Sigma). After washing with HBSS^{-/-}, tissues were digested using a lamina propria dissociation kit (Miltenyi) according to the manufacturer's instructions. Lymphocytes were further enriched on a 40%/80% Percoll (GE Healthcare) gradient.

Small intestines from fetal mice were isolated from embryos under a dissecting microscope. Surrounding mesenteric tissue was removed under further magnification and cut open longitudinally. Tissue was incubated for 30 min at 37°C under agitation in HBSS containing calcium and magnesium (HBSS^{+/+}), 2% FCS, 10 mM HEPES, 0.2 U/mL Liberase DL (Sigma) and 50 µg/mL DNaseI (Sigma). Leftover tissue was dissociated using a 22G needle and cell suspension was passed through a 70 µm strainer and washed in PBS/BSA. E14.5 and E16.5 time points were pooled samples; E18.5 intestines were treated separately.

Fetal liver was isolated under a dissecting microscope and a single cell suspension was generated by vigorously pipetting using a 1000 µL pipette. Cell suspension was filtered through a 70 µm strainer, washed in PBS/BSA followed by erythrocyte lysis.

Embryonic lymph node anlagen from the mesenteric region were dissected under a microscope or isolated from adult animals and passed through a 70 µm strainer.

Table 4. Tissue isolation reagents.

Reagent or resource	Source	Identifier
Miltenyi Si LPL Kit	Miltenyi	Cat. No. #130-097-410
HBSS ^{-/-}	Gibco	Cat. No. #14065-056
HBSS ^{+/+}	Gibco	Cat. No. #14065-056

HEPES	Sigma	Cat. No. #54457
DTT	ThermoFisher	Cat. No. #R0861
FCS	ThermoFisher	Cat. No. #16010159
EDTA	Sigma	Cat. No. #PHR2586
Percoll	GE Healthcare	Cat. No. #17-0891-01
Liberase DL	Sigma	Cat. No. #05466202001
DNaseI	Sigma	Cat. No. #DN25
PBS/BSA	In house	N/A
Gentle MACS™	Miltenyi	Cat. No. # 130-096-427

3.3 Flow cytometry

Cells were stained using the following antibodies: APC-Vio770 anti-mouse CD19 (REA749), APC-Fire780 anti-mouse F4/80 (BM8), APC-Fire780 anti-mouse Gr-1 (RB6-8C5), APC-Cy7 anti-mouse Fc ϵ RI α (MAR-1), BV510 or BUV396 anti-mouse CD45 (30-F11), APC anti-mouse CD45.1 (A20), VioGreen anti-mouse CD45.2 (104-2), PerCP-eF710 anti-mouse $\gamma\delta$ TCR (GL-3), A700 or PerCP-Cy5.5 anti-mouse β TCR (H57-597), PerCP-Vio700 anti-mouse CD3 (REA641), PE-Dazzle594 or BV605 or PE anti-mouse NKp46 (29A1.4), BV650, BV711, BV785 or PE-Cy7 or AlexaFlour647 anti-mouse CD4 (RM4-5), BV650 or BV711 anti-mouse CD8 (53-6.72), BV785 anti-mouse CD127 (A7R34), PE-Vio770 anti-mouse CD122 (REA1015), PE anti-mouse CCR6 (29-2L17), PE-Vio615 anti-mouse CXCR5 (REA215), PerCP-eF710 anti-mouse CXCR6 (DANID2), Alexa700 anti-mouse CD90 (T24), BV421 anti-mouse $\alpha_4\beta_7$ (DATK32), PE-Cy7 or BV605 anti-mouse KLRG1 (2F1/KLRG1), BUV661 or BV785 anti-mouse NK1.1 (PK136), PE-Vio770 anti-human/rat/mouse ICOS (REA192), BUV563 anti-mouse CD38 (90/CD38), BV711 anti-mouse IgD (11-26c.2a), PerCP-eF710 anti-mouse GL7 (GL7), PE anti-mouse Fas (Jo2), BV510 or BV711 or PE-Cy7 anti-mouse PD1 (29F.1A12). For staining with PE anti-mouse RANKL (IK22/5) and LT $\alpha_1\beta_2$ -hFab ([Lee et al., 2006](#)) and anti-hIgG APC (IS11-12E4.23.20) cells were incubated for 1 h at 37°C in complete RPMI medium containing 10% FCS. To minimize non-specific binding of antibodies, cells were blocked with anti-mouse CD16/32 (2.4G2) and dead cells were excluded by staining with Fixable Viability Dye (LD) (ThermoFisher) prior to staining.

For intranuclear staining, cells were fixed with transcription factor staining buffer set (BD Bioscience) according to the manufacturer's instructions. For fixation of fate-map YFP signal,

cells were fixed 20 min at room temperature in 2% para-formaldehyde (EMS) and stained in BD Bioscience perm buffer with PE-Vio615 or FITC anti mouse/human GATA3 (REA174), AlexaFluor647 anti-mouse T-bet (4B10), PE or BV650 anti-mouse ROR γ t (Q31-378), eFluor450 Eomes (Dan11mag), AlexaFluor647 anti-mouse/human PLZF (R17-809), APC anti-mouse Bcl-6 (REA373), A700 anti-mouse Ki67 (16A8). Unless otherwise stated all antibodies were purchased at Miltenyi, Themofisher, Biolegend UK or BD Biosciences.

For the assessment of IL-22 and IL17A-producing cells, single-cell suspensions obtained from small intestinal lamina propria were stimulated in 10% FCS IMDM and 10 ng/mL of PMA (Sigma-Aldrich) and 500 ng/mL ionomycin (Sigma-Aldrich) in the presence of GolgiPlug containing brefeldin A (BD Biosciences) for 4 h at 37°C. Following washing, cells were surface labelled with above listed antibodies and fixed in 1x InsideFix (Inside Stain Kit Miltenyi Biotec) for 20 min at ambient temperature. Intracellular staining using APC anti-mouse IL-22 (IL22JOP) and BV711 anti-mouse IL-17A (TCC11-18H10.1) was performed in InsidePerm (Inside Stain Kit Miltenyi Biotec) for 30 min at room temperature.

Flow cytometry was performed using a LSR Fortessa and BD FACSymphony flow cytometer (BD Biosciences). Data were further analysed with Flow Jo. All flow cytometry analysis was performed according to the Guidelines for the use of flow cytometry and cell sorting in immunological studies (Cossarizza et al., 2017).

Table 5. Flow cytometry resources.

Reagent or resource	Source	Identifier
APC-Vio770 anti-mouse CD19 (REA749)	Miltenyi	Cat. No. #130-112-038
APC-Fire780 anti-mouse F4/80 (BM8)	BioLegend	Cat. No. #123116
APC-Fire780 anti-mouse Gr-1 (RB6-8C5)	BioLegend	Cat. No. #108456
APC-Cy7 anti-mouse Fc ϵ RI α (MAR-1)	BioLegend	Cat. No. #134326
BV510/BUV396 anti-mouse CD45 (30-F11)	BioLegend/BD	Cat. No. #103138 / 564279
APC anti-mouse CD45.1 (A20)	Miltenyi	Cat. No. #130-102-470
VioGreen anti-mouse CD45.2 (104-2)	Miltenyi	Cat. No. #130-102-312
PerCP-eF710 anti-mouse $\gamma\delta$ TCR (GL-3)	ThermoFisher	Cat. No. #46-5711-82
A700/PerCP-Cy5.5 anti-mouse β TCR (H57-597)	BioLegend	Cat. No. #109224 / 109228
PerCP-Vio700 anti-mouse CD3 (REA641)	Miltenyi	Cat. No. #130-120-826

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BV605/PE/PE-Dazzle594 anti-mouse NKp46 (29A1.4)	BioLegend	Cat. No. #137619 / 137604 / 137629
BV650/BV711/PE-Cy7/AlexaFlour647/BV785 anti-mouse CD4 (RM4-5)	BioLegend	Cat. No. #100546 / 100550 / 100528 / 100530 / 100552
BV650/BV711 anti-mouse CD8 (53-6.72)	BioLegend	Cat. No. #100742 / 100759
BV785 anti-mouse CD127 (A7R34)	BioLegend	Cat. No. #135037
PE-Vio770 anti-mouse CD122 (REA1015)	Miltenyi	Cat. No. #130-117-013
PE anti-mouse CCR6 (29-2L17)	BioLegend	Cat. No. #129804
PE-Vio615 anti-mouse CXCR5 (REA215)	Miltenyi	Cat. No. #130-107-656
PerCP-eF710 anti-mouse CXCR6 (DANID2)	ThermoFisher	Cat. No. #46-9186-82
Alexa700 anti-mouse CD90 (T24)	In house	N/A
BV421 anti-mouse $\alpha_4\beta_7$ (DATK32)	BD	Cat. No. #566294
PE-Cy7/BV605 anti-mouse KLRG1 (2F1/KLRG1)	BioLegend	Cat. No. #138416 / 138419
BUV661/BV785 anti-mouse NK1.1 (PK136)	BD/BioLegend	Cat. No. #741477 / 108749
PE anti-mouse RANKL (IK22/5)	BioLegend	Cat. No. #510006
LT $\alpha_1\beta_2$ -hFab	(Lee et al., 2006)	N/A
anti-hIgG APC (IS11-12E4.23.20)	Miltenyi	Cat. No. #130-119-857
PE-Vio770 anti-mouse ICOS (REA192)	Miltenyi	Cat. No. #130-100-735
BUV563 anti-mouse CD38 (90/CD38)	BD	Cat. No. #741271
BV711 anti-mouse IgD (11-26c.2a)	BioLegend	Cat. No. #405731
PerCP-eF710 anti-mouse GL7 (GL7)	ThermoFisher	Cat. No. #46-5902-82
PE anti-mouse Fas (Jo2)	BD	Cat. No. # 554258
BV510 or BV711 anti-mouse PD1 (29F.1A12)	BioLegend	Cat. No. #135241 / 135231

PE-Cy7 anti-mouse PD1 (J43)	ThermoFisher	Cat. No. # 25-9985-82
BV421 anti-mouse ST2 (DIH9)	BioLegend	Nat. No. #145309
PE-Vio615/FITC anti mouse/human GATA3 (REA174)	Miltenyi	Cat. No. #130-109-115 / 130-120-147
AlexaFluor647 anti-mouse T-bet (4B10)	BioLegend	Cat. No. #644804
PE/BV650 anti-mouse ROR γ t (Q31-378)	BD	Cat. No. #562607 / 564722 / 562894
eFluor450 Eomes (Dan11mag)	ThermoFisher	Cat. No. #48-4875-82
AlexaFluor647 anti-mouse/human PLZF (R17-809)	BD	Cat. No. #563490
APC anti-human/mouse Bcl-6 (REA373)	Miltenyi	Cat. No. #130-121-997
A700 anti-mouse Ki67 (16A8)	BioLegend	Cat. No. #652420
anti-mouse CD16/32 (2.4G2)	In house	N/A
Fixable Viability Dye (LD)	ThermoFisher	Cat. No. #65-0865-18
transcription factor staining buffer set	ThermoFisher	Cat. No. #00-5523-00
para-formaldehyde	EMS	Cat. No. #50-980-488
LSR Fortessa	BD	https://www.bdbiosciences.com/en-eu/instruments/research-instruments/research-cell-analyzers/lrsfortessa
BD FACSymphony flow cytometer	BD	https://www.bdbiosciences.com/en-eu/instruments/research-instruments/research-cell-analyzers/facsymphony
FlowJo Software v10	FlowJo	https://www.flowjo.com/solutions/flowjo

3.4 *In vitro* cell culture

Single-cell suspensions from E14.5 fetal liver were enriched for CD45⁺ cells using magnetic activated cell sorting, followed by staining with fluorochrome-conjugated antibodies and sorted on BD FACS Aria II (BD Bioscience) as live Lin⁻CD45⁺Flt3⁻CD127⁺ $\alpha_4\beta_7$ ⁺PD1⁻ cells. Sorted lymphocytes were subsequently resuspended in IMDM (ThermoFisher) supplemented with 10% FCS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 50 μ M β -mercaptoethanol (ThermoFisher) and each 20 ng/mL recombinant mouse IL-7 and SCF. Cells were plated onto 70% confluent OP9 stromal cells in round bottom 96-well plates and medium was renewed every three days by replacing half of the media. Cells were analysed by flow cytometry after 5-8 days of culture.

Table 6. *In vitro* cell culture reagents.

Reagent or resource	Source	Identifier
OP9	ATCC	CRL2749
CD45 microbeads	Miltenyi	Cat. No. #130-052-301
IMDM	ThermoFisher	Cat. No. #12440053
Penicillin-Streptomycin	ThermoFisher	Cat. No. # 15140148
β -mercaptoethanol	ThermoFisher	Cat. No. #21985023
IL-7, research grade	Miltenyi	Cat. No. #130-094-066
SCF, research grade	Miltenyi	Cat. No. #130-094-079
96 well cell culture plate, U-bottom	Greiner bio-one	Cat. No. #650180
CO ₂ incubator	Binder	Cat. No. #9040-0039
BD FACS AriaII cell sorter	BD	https://www.bdbiosciences.com/en-us/products/instruments/flow-cytometers/research-cell-sorters/bd-facsaria-iii

3.5 Adoptive transfer

For adoptive transfer experiments, *Rag2*^{-/-}*Il2rg*^{-/-} CD45.1 recipient mice (Thy1.2/1.2) were sublethally irradiated with 5 Gy and endogenous, irradiation-resistant ILC3 populations were

furthermore depleted by i.p. administration of 250 µg anti-mouse Thy1.2 (30H12, in-house production) every third day. E14.5 FL lymphoid progenitors were FACS-sorted on an AriaII (BD Biosciences) instrument using the following sorting strategy LinLD⁻CD45⁺CD127⁺. CD45.2⁺ donor cells (Thy1.1/1.1 congenic) were injected i.v. (**Figure 8**). Mice were kept under antibiotic treatment in the first two weeks and analyzed 4 weeks after transfer.

Figure 8. Schematic overview of adoptive transfer experimental setup. CD45.1⁺CD90.2⁺ congenic *Rag2*^{-/-}*Il2rg*^{-/-} hosts were irradiated and additionally anti-CD90-depleted every third day from d-1 onwards. E14.5 fetal liver (FL)-derived progenitors of CD45.2⁺CD90.1⁺ donor reporter (*Rorc(gt)*^{GFP/wt}), RKO (*Rorc(gt)*^{GFP/GFP}) and DKO (*Rorc(gt)*^{GFP/GFP} *Tbx21*^{-/-}) mouse strains were i.v. injected and host mice were analysed 4 weeks after reconstitution.

3.6 Lymph node enumeration

Percentages of mice with LN were calculated for each LN type using the total number of mice with the respective LN found in a given strain relative to the maximal number of mice with LNs of that type found in *Rorc(gt)*^{GFP/wt} or *Il7*^{cre} control mice.

3.7 Immunofluorescence staining

Lymph nodes were fixed for 4 h in 4% paraformaldehyde and dehydrated via a sucrose gradient for 24 h, embedded in O.C.T. compound and frozen in 2-Methylbutane within ethanol rinsed dry ice bath. Frozen lymph nodes were cryotome-cut into 7 µm sections and placed on microscope slides in pairs. After thawing and PBS-rehydration of slides, primary staining was carried out after 20 min blocking with 10% goat serum in PBS/BSA-0.1%Tween20 (P-B-T). DAPI, anti-IgD-AF594, PNA-bio and rat-anti-mouse-CD35 were incubated for 1 h in P-B-T. Secondary staining was performed after washing in P-B-T by 30 min incubation with SA-A555 and anti-rat-IgG-A647 in P-B-T. Samples were finally washed in PBS and covered with mounting medium and cover slip and before image acquisition was performed on Zeiss LSM 880 confocal microscope.

Table 7. Histology resources.

Reagent or resource	Source	Identifier
2-Methylbutane	Carl Roth	Cat. No. #3997.1
4',6-Diamidino-2-phenyl-indol -dihydrochlorid	Sigma-Aldrich	Cat. No. #D9542
Anti-mouse-CD35 unlabeled, rat IgG2a, kappa (8C12)	BD	Cat. No. #558768
Anti-rat-IgG-Alexa Fluor647, Goat IgG polyclonal	ThermoFisher	Cat. No. #A-21247
Anti-mouse-IgD-Alexa Fluor594, Rat IgG2a, kappa (11-26c.2a)	BioLegend	Cat. No. #405740
Peanut-agglutinin, biotynilated	Vector Labs	Cat. No. #B-1075-5
Streptavidin-Alexa-Fluor555	Invitrogen by Thermo Fisher Scientific	Cat. No. #S32355
Ethanol	In-house	N/A
Fluoromount Aqueous Mounting Medium	Sigma-Aldrich	Cat. No. #F4680
Paraformaldehyde	Electron Microscopy Sciences	Cat. No. #15713
Tissue-Tek O.C.T. compound	Sakura	Cat. No. #4583
Goat serum	Gibco	Cat. No. #16210-064

3.8 Quantitative real-time PCR

mRNA was isolated from small intestinal intraepithelial cells by using NucleoSpin® RNA Mini Kit (Macherey-Nagel) and following the manufacturer's protocol. RNA integrity was assessed on a

BioAnalyzer (Agilent) and RNA samples with a RIN score ≥ 7 were further transcribed into first strand cDNA by using Reverse Transcription Reagents (Applied Biosystems). qPCR reactions were assayed in triplicates per sample by using a StepOne Plus real-time PCR system and TaqMan Gene expression assays (all ThermoFisher): Reg3g (Mm00441127_m1), Reg3b (Mm00440616_g1), Gapdh (Mm99999915_g1), HPRT (Mm03024075_m1) and Actb (Mm02619580_g1). mRNA content was normalized relative to the mean expression of the arithmetic means of Gapdh, Hprt and Actb CT values by applying the comparative C_T method ($2^{-\Delta C_T}$) in which $\Delta C_T (\text{gene of interest}) = C_T (\text{gene of interest}) - C_T (\text{arithmetic mean of housekeeping reference value})$.

Table 8. Commercial qPCR reagents.

Reagent or resource	Source	Identifier
NucleoSpin® RNA Mini Kit	Macherey-Nagel	Cat. No. #740955.50
TaqMan™ Reverse Transcription Reagents	Invitrogen	Cat. No #N8080234
TaqMan Assay FAM-MGB: Reg3g	ThermoFisher	Assay ID #Mm00441127_m1
TaqMan Assay FAM-MGB: Reg3b	ThermoFisher	Assay ID #Mm00440616_g1
TaqMan Assay FAM-MGB: Gapdh	ThermoFisher	Assay ID #Mm99999915_g1
TaqMan Assay FAM-MGB: HPRT	ThermoFisher	Assay ID #Mm03024075_m1
TaqMan Assay FAM-MGB: Actb	ThermoFisher	Assay ID #Mm02619580_g1
BioAnalyzer 2100	Agilent	Cat. No #G2939BA
Bioanalyzer High Sensitivity RNA 6000 Nano Kit	Agilent	Cat. No #5067-1511
StepOne Plus real-time PCR system	ThermoFisher	Cat. No #4376600

3.9 RNA sequencing and data analysis

RNA of cells was extracted using RNeasy Plus Micro Kit (Qiagen) from sorted small intestinal lamina propria cells using the following markers LinLD⁻CD45⁺CD3⁻RORγt⁺-GFP⁺ expressing either NKp46 or CD4. RNA quality was assessed by an Agilent 2100 Bioanalyzer. SMART-SeqII (ultra-low input RNA) libraries were prepared using Nextera XT DNA sample preparation kit (Illumina). Sequencing was performed on an Illumina HiSeq4000 platform, PE100. For heatmaps, normalized RNA-seq data z-score transformed were plotted using ggplot2 R package. RNA-seq datasets analysed are publicly available in Gene Expression Omnibus repository with the accession number GSE161439.

Table 9. Bulk seq reagents.

Reagent or resource	Source	Identifier
RNeasy Plus Micro Kit	Qiagen	Cat. No #74034
Nextera XT DNA library preparation kit	Illumina	Cat. No #FC-131-1024
BioAnalyzer 2100	Agilent	Cat. No #G2939BA
Bioanalyzer High Sensitivity RNA 6000 Pico Kit	Agilent	Cat. No #5067-1513
HiSeq4000 platform	Illumina	https://www.illumina.com/systems/sequencing-platforms/hiseq-3000-4000.html

3.10 10x Genomics Chromium single-cell RNA-sequencing

Fetal small intestinal lymphocytes were isolated as described above and sorted as LinLD⁻CD45⁺CD122⁺ and/or CD127⁺. A total of 7 embryos from one pregnant *Rorc(gt)^{GFP/wt}* female, 18 embryos from two pregnant *Rorc(gt)^{GFP/GFP}* females and 19 embryos from three pregnant *Rorc(gt)^{GFP/wt} × Tbx21^{-/-}* females were used. Samples were furthermore labelled with antibody-derived tags against specific extra-cellular targets and genotypes hash-tagged with TotalSeq™-A antibodies (BioLegend) following the manufacturer's protocol. A total of 8000 cells were loaded in the Chromium™ Controller for partitioning single cells into nanoliter-scale Gel Bead-In-Emulsions (GEMs). Single Cell 3' reagent kit v3.1 was used for reverse transcription, cDNA amplification and library construction of gene expression libraries (10x Genomics) according to the manufacturer's instructions. TotalSeq™-A libraries were prepared following the protocol for 10x Single Cell 3' Reagent Kit v3.1 provided by BioLegend, including primer sequences and

reagent specifications. Concentrations of all libraries were quantified using a Qubit™ 2.0 Fluorometer (ThermoFisher) and quality was tested on a 2100 Bioanalyzer with High Sensitivity DNA kit (Agilent). Sequencing was performed using the NextSeq500/550 HighOutput Kit v2.5 (150 cycles) on a NextSeq500 sequencer (Illumina). scRNA-seq datasets analyzed are publicly available in Gene Expression Omnibus repository with the accession number GSE161441.

Table 10. ScRNA-seq reagents.

Reagent or resource	Source	Identifier
Anti-mouse CD49b (HMa2)	BioLegend	Cat. No. #103523
Anti-mouse NK1.1 (PK136)	BioLegend	Cat. No. #108755
Anti-mouse DNAM1 (10E5)	BioLegend	Cat. No. #128823
Anti-mouse CD3 (17A2)	BioLegend	Cat. No. #100251
Anti-mouse CCR6 (29-2L17)	BioLegend	Cat. No. #129825
Anti-mouse Nkp46 (29A1.4)	BioLegend	Cat. No. #137633
Anti-mouse CD117 (2B8)	BioLegend	Cat. No. #105843
Anti-mouse Nrp-1 (3E12)	BioLegend	Cat. No. #145215
Anti-mouse NKG2D (CX5)	BioLegend	Cat. No. #130215
Anti-mouse TCRgd (GL3)	BioLegend	Cat. No. #118137
Anti-mouse TCRb (H57-597)	BioLegend	Cat. No. #109247
Anti-mouse CD49a (HMa1)	BioLegend	Cat. No. #142613
Anti-mouse CXCR5 (L138D7)	BioLegend	Cat. No. #145535
Anti-mouse CD27 (LG.3A10)	BioLegend	Cat. No. #124235
Anti-mouse CD200R1 (OX-110)	BioLegend	Cat. No. #123913
Anti-mouse CD4 (RM4-5)	BioLegend	Cat. No. #100569
Anti-mouse PD-1 (RMP1-30)	BioLegend	Cat. No. #109123
Anti-PE(PE001)	BioLegend	Cat. No. #408109
anti-Biotin (1D4-C5)	BioLegend	Cat. No. #409008

Anti-mouse ICOS (C398.4A)	BioLegend	Cat. No. #313555
Anti-mouse Sca-1 (D7)	BioLegend	Cat. No. #108147
Anti-mouse ST2 (DIH9)	BioLegend	Cat. No. #145317
Anti-mouse CD25 (PC61)	BioLegend	Cat. No. #102055
Anti-mouse KLRG1 (2F1/KLRG1)	BioLegend	Cat. No. #138431
Hashtag A0301 (M1/42; 30-F11)	BioLegend	Cat. No. #155801
Hashtag A0302 (M1/42; 30-F11)	BioLegend	Cat. No. #155803
Hashtag A0303 (M1/42; 30-F11)	BioLegend	Cat. No. #155805
Single Cell 3' reagent kit v3.1	10x Genomics	Cat. No. # PN-1000121
Chromium™ Controller	10x Genomics	https://www.10xgenomics.com/instruments/chromium-controller
Qubit™ 2.0 Fluorometer	ThermoFisher	N/A
Qubit™ dsDNA HS Assay Kit	ThermoFisher	Cat. No #Q32854
BioAnalyzer 2100	Agilent	Cat. No #G2939BA
Bioanalyzer High Sensitivity DNA Analysis	Agilent	Cat. No #5067-4626
NextSeq500/550 HighOutput Kit v2.5	Illumina	Cat. No #20024907
NextSeq500 sequencer	Illumina	N/A

3.11 scRNA-seq data processing

Demultiplexing and alignment to the mm10 reference transcriptome was performed with Cell Ranger v3.0.2 (Zheng et al., 2017). Antibody- and hashtag-barcodes were counted with CITE-seq-Count (Roelli et al., 2019) and normalized by centered log ratio transformation with Seurat v3.0.2 (Stuart et al., 2019), which was also used for further data processing. Genotypes were assigned based on normalized hashtag counts. Cells were filtered by number of transcripts, genes, percent mitochondrial genes and for cells present within transcriptome, antibody and hashtag libraries. Normalized expression values were generated with scan (Lun et al., 2016),

followed by scaling and principal component (PC) analysis. PC loadings and explained variance were visualized to determine PCs used for dimensionality reduction by uniform manifold approximation (UMAP) (McInnes et al., 2018) and Seurat clustering. Wilcoxon test was applied for differential gene expression analysis of genes expressed by $\geq 10\%$ of cells in a cluster and with a log fold change of ≥ 0.25 . Lineage structures on UMAPs were analyzed with Slingshot (Street et al., 2018), giving the starting (CLP) and end populations (ILC1/2/3) as input. Partition-based graph abstraction (PAGA) using the Fruchterman & Reingold algorithm was performed within Scanpy v1.4.6 (Wolf et al., 2019) after import of pre-processed Seurat objects into Python with anndata2ri (A. et al., 2020). scRNA-seq data analysis was performed with R v4.0.0 and Python v3.7.6. Gene modules of specific subsets from cells of *Rorc(gt)^{GFP/wt}* mice were defined by the top 100 differentially regulated genes calculated from the comparison of all cells from all subsets.

Table 11. ScRNA-seq data processing algorithms.

Reagent or resource	Source	Identifier
Cell Ranger v3.0.2	(Zheng et al., 2017)	https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/using/count
CITE-seq-Count	(Roelli et al., 2019)	
R Studio v4.0.0		https://rstudio.com/
Seurat v3.0.2	(Stuart et al., 2019)	
Scran	(Lun et al., 2016)	
Slingshot	(Street et al., 2018)	
Scanpy v1.4.6	(Wolf et al., 2019)	
Python v3.7.6		https://www.python.org/
Anndata2ri	(A. et al., 2020)	https://zenodo.org/record/3992373

3.12 Statistical analysis

Kruskal–Wallis test with Dunn’s multiple comparison correction was employed for statistical analysis of datasets. Statistical analyses were performed with Prism 5.04 (GraphPad Software)

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using a confidence level of 0.95, and P values >0.05 were considered not significant, P values from 0.01 to 0.05 were considered as *, P values from 0.001 to 0.005 were considered as **, P values <0.0001 were considered as ***.

Table 12. Softwares and algorithms.

Reagent or resource	Source	Identifier
GraphPadPrism 5.04	GraphPad Software Inc	https://www.graphpad.com/scientific-software/prism/
FlowJo 10	FlowJo LLC	https://www.flowjo.com/solutions/flowjo
R Studio v4.0.0	R Core Team	https://rstudio.com/
Python v3.7.6	Python Software Foundation	https://www.python.org/

4 Results

4.1 Identification of fetal ILC subsets in the developing mouse intestine

In order to study embryonic ILCs, the expression of the commonly used inclusion marker CD127 or IL-7R (Vonarbourg and Diefenbach, 2012) was characterized in the fetal small intestinal (SI) lamina propria 18.5 days post conception (E18.5). After exclusion of dead and lineage (CD19, Fc ϵ RI α , GR-1, F4/80, CD3)⁺ cells and inclusion of CD45⁺ cells of hematopoietic origin, CD127 was found to be highly expressed on ROR γ t⁺ ILC3, lower on GATA3^{hi} ILC2 and lowest on T-bet⁺ ILC1/NK. The usage of an additional marker CD122 (also known as IL-2R β), a member of the type I cytokine receptor family, that was highly expressed on T-bet⁺ ILC1/NK enabled us to incorporate all members of the ILCs including T-bet-expressing ILC1 (Figure 9 A, B). Further validating the gating strategy, additional ILC markers CXCR6 as well as CD90 were also expressed by all subsets and can be used to study embryonic intestinal ILCs (Figure 9 B, C). Gating on CD122⁺CD127⁺ cells revealed absence of CXCR6⁻, CD90⁻ as well as ROR γ t⁻, T-bet⁻ and GATA3⁻ expressing cells, emphasizing the approach of using not only CD127 but also CD122 as valuable inclusion markers for ILC identification in the embryonic gut.

Figure 9. Identification of embryonic ILCs by flow cytometry. **(A)** Flow cytometric representative plots showing expression of transcription factors on CD127⁺ and/or CD122⁺ on LinLD⁺CD45⁺ cells of the E18.5 small intestine (SI). **(B)** Specific ILC populations are defined by transcription factors within CD127⁺ and/or CD122⁺ cells that furthermore express the surface markers CXCR6 or CD90. **(C)** Within CD127⁺ CD122⁺ minor populations of ROR γ t, T-bet or GATA3 are detected.

4.2 Single-cell sequencing of fetal intestinal cells reveals a spectrum of mature ILC subsets and progenitors

For the purpose to study prenatal ILC heterogeneity, single-cell RNA sequencing (scRNA-seq) of viable Lin(CD19, CD3, CD5, F4/80, Fc ϵ RI α , Gr-1)⁻ CD45⁺ cells expressing CD127 and/or CD122 isolated from the small intestine (SI) of embryonic day 18.5 (E18.5) of *Rorc(gt)^{GFP/wt}* mice was performed.

For simultaneous characterization of RNA and protein, oligo-conjugated antibodies were integrated into the single-cell sequencing workflow, also known as CITE-seq (Cellular Indexing of Transcriptomes and Epitopes by sequencing) that enabled validation of ILC lineage identities with surface protein expression. After quality control, normalization and filtering out a DC-like population (displaying transcripts for *Cd209a*, *Cd86* and *Cfs1r*, **Figure 10 A**), we analysed 1268

remaining cells. Clearly distinct gene signatures marked by 498-1466 differentially expressed genes (DEGs) led to the identification of eight distinct clusters (**Figure 10 B and Figure 11 A**).

Figure 10. Single-cell RNA sequencing of E18.5 intestinal cells identifies distinct clusters. Viable Lin(CD19, CD3, CD5, F4/80, FcεRIα, Gr-1)- CD45+ cells expressing IL-7 receptor (CD127) and/or the IL-2 receptor subunit beta (CD122) isolated from the small intestine (SI) of E18.5 *Rorc(gt)^{GFP/wt}* embryos were sort-purified by flow cytometry, and a single-cell expression library was generated using 10x Genomics. **(A)** Violin plots of selected dendritic cell (DC)-associated markers. **(B)** Heatmap displaying the top 50 differentially expressed genes (DEGs) within each cluster, gene examples are given.

The key ILC marker *Id2* was transcribed in the majority of cells assigning these cells to the ILC lineage, except for one small cluster 8 (**Figure 11 B, D**). Besides *Id2*, other fundamental ILC transcription factors like *Ets1*, *Rora* or *Ahr* transcripts were detected (**Figure 11 D**). Strong gene signatures, including transcripts for *Gata3*, *Tbx21* and *Rorc*, facilitated the identification of three major superclusters comprising of ILC2 (cluster 1 and 7), of ILC1/NK (hereafter referred to as ILC1, cluster 3 and 5) and of ILC3/LTi (hereafter referred to as ILC3, cluster 2 and 6) (**Figure 11 A, B, D**). Identity of the inferred superclusters was also confirmed by the distribution of surface markers detected by oligo-conjugated antibodies as well as flow cytometry, ST2, NK1.1 and CD4 (**Figure 11 B, C**). Top 50 differentially expressed genes between these clusters included *Bcl11b*, *Il1rl1*, *Il17rb*, *Icos*, *Hey1*, *Il9r* and *Il4* for ILC2; *Ccl5*, *Klrk1*, *Ncr1*, *Cxcr3*, *Xcl1* and *Ifng* for ILC1; *Batf3*, *Batf*, *Tox2*, *Il1r1*, *Il23r*, *Nrp1*, *Lta* and *Il22* for ILC3 (**Figure 10 B and Figure 11 D**) (Gury-BenAri et al., 2016; Pokrovskii et al., 2019; Robinette et al., 2015). As also observed on protein level, transcripts for *Il7r* were highest in ILC3 and lowest on ILC1/NK, which conversely showcased most abundant *Il2rb* transcripts (**Figure 11 B, D**). Within all ILC superclusters, we further detected clusters of cells (5-7) enriched in gene transcripts associated to active cell cycle, such as *Mki67*, *Top2a* and *Tubb4*, highlighting a substantial fraction of proliferating ILC3, ILC2 and ILC1 within the embryonic intestine (**Figure 10 B and Figure 11 A**). Expression of the NK cell TF *Eomes* was mostly negative and limited to a small subset within *Tbx21*⁺ cells, supporting the notion that clusters 3 and 5 mainly comprise of ILC1 (**Figure 11 D, E**). In addition to classical ILC lineages, two other clusters (4 and 8) were observed of which cluster 8 lacked expression of most ILC-related genes such as *Id2* but displayed abundant transcripts for *Flt3*, *Cd34* and *Bcl11a* along with *Il7r* (**Figure 11 B, D**). Moreover, transcripts for *Tcf3* (encoding for E2A) which facilitates lymphocyte differentiation from hematopoietic stem cells but also *Nfil3* transcripts were found, a transcription factor that controls NK/ILC lineage commitment by regulating *Id2* expression (Male et al., 2014; Seillet et al., 2014b, 2014a; Xu et al., 2015). Few cells within this cluster additionally showcased expression of recombination activating genes *Rag1* or Early B cell Factor 1 (*Ebf1*), altogether designating this expression signature being compatible with the one previously described for common lymphocyte progenitors (CLP) from adult BM (Ghaedi et al., 2016). Cluster 4 on the other hand showed high expression of most ILC lineage-associated genes such as *Id2*, *Ets*, *Rora* or *Gata3* but also had abundant transcripts for *Zbtb16* encoding for PLZF, *Tcf7* or *Tox*, core regulators of multiple ILC developmental pathways (Constantinides et al., 2014; Klose et al., 2014; Mielke et al., 2013; Seillet et al., 2016; Yang et al., 2013). Transcripts such as *Ikzf2*, *Runx3* and *Maf*, genes that are implied in commitment to ILC lineages were similarly upregulated in this cluster suggesting that these cells are enriched in ILC precursors (ILCP), sharing a gene signature previously described in FL and adult BM (**Figure 11 D**). Thus, cluster 8 and 4 are hereafter referred to as CLP and ILCP, respectively. Interestingly, a conversion from *Notch1* to *Notch2* expression was noted in

these two progenitor clusters underlining the transition from CLP to ILCP as reported in FL progenitors by Golub et al. (Chea et al., 2016b).

*legend on next page

Figure 11. Single-cell RNA sequencing of fetal intestinal cells reveals a spectrum of mature ILC subsets and progenitors. Viable Lin(CD19, CD3, CD5, F4/80, FcεRIα, Gr-1)⁻ CD45⁺ cells expressing IL-7 receptor (CD127) and/or the IL-2 receptor subunit beta (CD122) isolated from the small intestine (SI) of E18.5 *Rorc(gt)^{GFP/wt}* embryos were sort-purified by flow cytometry, and a single-cell expression library was generated using 10x Genomics. **(A)** UMAP dimensional reduction projection identifies 8 distinct clusters. **(B)** Gene expression and CITE-seq protein expression UMAP plots. **(C)** representative flow cytometry plots identifying ST2 expression on GATA3⁺ ILC2, NK1.1 expression on T-bet⁺ NK/ILC1 and CD4 expression by RORγt-GFP⁺ ILC3. **(D)** Selected gene expression within clusters. Colour scale represents average expression, dot size visualizes fraction of cells within the cluster expressing the gene. **(E)** UMAP-projected expression of *Eomes* transcripts.

ILCP mostly co-expressed *Zbtb16* together with *Tcf7*, *Tox* and also *Gata3^{lo}*, while few cells co-expressed *Zbtb16* with *Cxcr6*, *Gata3^{hi}*, *Tbx21* or *Rorc* with only little or no expression in CLP **(Figure 12 A)**, suggestive of continuous transition from ILCP towards the diverse ILC lineages. On this matter, expression of genes linked to specific lineages such as ILC1-associated transcripts *Cxcr3*, *Klrk1*, *Xcl1*, as well as ILC2-associated transcripts *Il17rb*, *Icos*, *Il4*, and ILC3-associated transcripts *Batf3*, *Batf*, *Nrp1* and *Il1r1* could be observed accordingly within the ILCP cluster **(Figure 11 D)**. In line with this, surface PD-1 expression, which was shown to identify PLZF⁺ ILCP in BM (Seillet et al., 2016; Yu et al., 2016) correlated with *Zbtb16* transcripts. Fetal intestinal ILCP were also characterized by the expression of the heterodimeric integrin receptor α4β7 and c-Kit, as previously shown for their BM and FL counterpart **(Figure 12 B)**.

In order to test whether the transcriptional signatures displayed by fetal intestinal ILC and progenitors were compatible with a model of in situ differentiation from CLP, via ILCP, to mature ILC subsets single-cell developmental trajectories were inferred, using two distinct computational models, namely partition-based graph abstraction (PAGA) **(Figure 12 C)** (Wolf et al., 2019) and Slingshot **(Figure 12 D)** (Street et al., 2018). For this analysis, cell-cycle related genes were regressed out to correct the positioning of the proliferating clusters (5, 6, 7) in the embedding, thereby mapping these cells onto their respective ILC superclusters. Both analyses revealed comparable trajectories with continuous cell transitions from CLP to ILCP, further branching into ILC1, ILC2 and ILC3. Of note, Slingshot analysis emphasized an early branching of the ILCP towards the ILC2 cluster, while ILC1 and ILC3 retained a common trajectory and transitional states before bifurcating into separate lineages **(Figure 12 D)**. Pseudotime analysis of selected genes showed early expression of *Zbtb16*, *Tcf7* and *Tox* where ILC1/NK upregulate *Zbtb16* and ILC3 upregulate *Tcf7* after lineage commitment **(Figure 12 E)**. Notably, ILC3 lineage shows lowest expression of *Zbtb16* but highest expression of *Tcf7* suggesting a potential regulation of ILC3 differentiation by these two TFs. Additional evaluation of *Il1r1*, *Ncr1* and *Il1r1* transcripts in ILC2, ILC1 and ILC3 lineages respectively indicated upregulation of these genes later in pseudotime and validated the analysis **(Figure 12 E)**.

Figure 12. Single-cell RNA sequencing defines CLP and ILCP progenitors within the embryonic intestine. (A) *Zbtb16* co-expression plots of selected genes within CLP and ILCP cluster. (B) Gene expression and CITE-seq protein expression UMAP plots. (C) Trajectory analysis using PAGA lineage interference method. (D) Trajectory analysis using Slingshot. Inferred trajectories are represented as lines starting within the CLP cluster. Dots represent knots. (E) Selected gene expression over pseudotime within ILC1, ILC2 and ILC3.

Altogether, these data report prenatal transcriptional signature of ILC lineages in SI lamina propria and identify progenitor populations within the isolated tissue that resemble those described in FL or adult BM, highlighting the fetal intestine as an active niche for ILC differentiation.

4.3 Transcriptomic profiling on single-cell level discloses heterogeneity within the embryonic ILC3 supercluster

Next, the ILC3 cluster was further dissected to assess whether fetal ILC3 comprise heterogeneous subsets similar to their adult counterparts. Using unsupervised clustering, three subclusters with defined sets of 350-496 differentially regulated genes were identified (Figure 13 A and B), highlighting heterogeneity within this fetal ILC lineage, despite comparable expression of the ILC3 TF *Rorc* in all three subclusters (Figure 13 C). Among the top 30 genes characterizing subcluster 1, *Tnfrsf11* (encoding RANKL or TRANCE), *Pdcd1* and transcripts for

cytokines or chemokines such as *Il22*, *Lif* (Cella et al., 2009), *Csf2* (encoding GM-CSF) and the neutrophil chemoattractant *Cxcl2* (Figure 13 C, D) were found. *Il7r*, *Ltb*, *Ccr6*, *Batf* and *Cd7* were among the transcripts enriched in subcluster 2. Interestingly, expression of transcripts associated with major histocompatibility (MHC) class molecules, including H2-genes as well as *Cd74*, *Cd82* and *Lst1*, previously reported in CD4⁺ fetal LTi cells and in CCR6⁺ ILC3 postnatally (Von Burg et al., 2014; Hepworth et al., 2013; Lehmann et al., 2020; Mackley et al., 2015; Mebius et al., 1997; Robinette et al., 2015), were specifically upregulated in this subcluster (Figure 13 B). Subcluster 3 showcased augmented expression of genes typically associated to T-bet-expressing ILC1 and NK cell subsets, as well as adult NKp46⁺ ILC3, such as *Klrk1*, *Klrc2*, *Klrd1*, *Klrb1c* together with *Nkg7* (Figure 13 C, D). Overall, these data suggest that generation of specified ILC3 subsets already happens before birth. Therefore, selected gene and protein expression patterns indicative of T-bet expression were analyzed in the next step to find out whether these cells could potentially be distinguished by surface markers.

Figure 13. Gene expression patterns of specified embryonic ILC3 subsets defined by scRNA-seq. (A) UMAP dimensional reduction projection reveals three separate subclusters within the ILC3 cluster. (B) Heatmap displaying the top 30 differentially expressed genes (DEGs). (C) Violin plot of *Rorc* expression within ILC3 subclusters. (D) Selected gene expression UMAP plots.

Indeed, cell surface stainings of c-Kit and DNAM-1 protein correlated with enriched transcripts and displayed differential surface binding (**Figure 14 A**). Hence, these markers could be used to characterize prenatal ILC3 subsets similar to how they were applied to differentiate between subsets in adult mice (Verrier et al., 2016). Besides aforementioned enriched transcripts of LTI-associated gene *Tnfrsf11* in subcluster 1, also *Cxcr5* and *Lta* were among the DEGs (**Figure 14 B**) suggesting that this subset represents cells capable of inducing LN formation. Importantly,

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protein expression of CD4 or NRP-1, surface receptors previously reported to identify LTi cells in embryos or adult mice and humans (Robinette et al., 2015; Shikhagaie et al., 2017), did not resolve cluster 1 and 2 (**Figure 14 B**). Additionally, cells expressing transcripts for *Tbx21* as well as for *Ncr1* (encoding for NKp46) were detected (**Figure 14 C**) and enriched in subcluster 3. In line with low expression of CD127 protein observed in T-bet-expressing ILCs (**Figure 14 D**), *Il7r* transcripts as well as CD127 protein were lowest in this subcluster of ILC3 co-expressing T-bet (**Figure 9 A and B**).

Figure 14. Transcriptomic profiling on single-cell level discloses heterogeneity within the embryonic ILC3 supercluster. (A, B) Violin plots of expressed transcripts or proteins within ILC3 subclusters. **(C)** Co-expression plots of *Tbx21* and *Ncr1* within ILC3 subclusters. **(D)** Violin plots of *Il7r* transcripts or CD127 protein within ILC3 subclusters.

4.4 A subset of ROR γ t⁺T-bet⁺CD4⁺ ILC3 emerges at E14.5 during embryonic development

To validate the scRNA-seq data by flow cytometry, bacterial artificial chromosome (BAC) transgenic T-bet-ZsGreen reporter mice were used to accurately detect T-bet expressing ILC by flow cytometry. Further, to monitor when T-bet⁺ ILC3 emerge during embryonic development, a kinetic analysis of T-bet and ROR γ t expression by LinLD⁻CD45⁺CD122⁺ and/or CD127⁺ cells derived from the developing SI or FL was performed. First, T-bet-ZsGreen reporter signal was validated by intracellular staining of T-bet, showing full co-expression of T-bet and ZsGreen fluorescent proteins (**Figure 15 A**). Both ROR γ t⁺ ILC3 and T-bet⁺ ILC1/NK were present in SI and liver of E14.5 mice (**Figure 15 B and C**) where T-bet⁺ subsets mainly comprised of ILC1 since Eomes was not detected (**Figure 15 D, F, G**). Within ROR γ t⁺ ILC3, classical CD4⁺ and

CD4⁺T-bet⁻ LTi cells could be observed, as described previously (Eberl et al., 2004; Mebius et al., 2001). Besides classical LTi cells, around 25% of ROR γ t⁺ ILC3 co-expressed T-bet and a fraction of these cells co-expressed CD4 (**Figure 15 B and C**). Similar subsets of ILC3 were dissected according to T-bet and CD4 expression were also observed in the E14.5 mesenteric LN (mLN) anlage (**Figure 15 E**). Further characterization of intestinal (**Figure 15 F**) and liver (**Figure 15 G**) ILC3 subsets showed high surface expression of CD127, NRP-1 and CXCR5 in the CD4⁺T-bet⁻ subset, with intermediate levels in CD4⁺T-bet⁺ and lowest expression in CD4⁻T-bet⁺ cells. All ILC3 subsets expressed high levels of $\alpha\alpha\beta\gamma$; conversely, CD90 was enriched in T-bet⁻ compared to T-bet⁺ ILC3 at E14.5 (**Figure 15 F and G**).

Figure 15. A subset of ROR γ t⁺T-bet⁺CD4⁺ ILC3 emerges during embryonic development. Flow cytometric analysis of E14.5 T-bet ZsGreen reporter mice. **(A)** Representative verification of T-bet protein and ZsGreen reporter signal in E14.5 small intestine (SI) or liver. Representative gating of E14.5 SI **(B)** or liver **(C)** for identification of T-bet⁺ROR γ t⁺ ILC1 (grey shaded gate) and ROR γ t⁺ ILC3. **(D)** Representative plots of Eomes expression in E14.5 SI and liver. **(E)** Representative gating of E14.5 mLN anlagen (pooled sample) for identification of T-bet⁺ROR γ t⁺ ILC1 and ROR γ t⁺ ILC3 subsets. Representative histograms of marker expression by ILC1/NK or ILC3 subsets based on expression of CD4 and T-bet ZsGreen from E14.5 SI **(F)** or liver **(G)**. Data are representative of 2-3 independent experiments.

We next comparatively analyzed the kinetic of ILC subsets in SI and FL to understand whether frequencies of these populations varied in time pre- and postnatally. While the percentage of

LinLD⁻CD45⁺CD122⁺ and/or CD127⁺ cells was generally lower in FL than in SI (**Figure 15 B and C**), ILC3 represented the majority in SI and ILC1 were present at lower frequencies within group 1/3 ILCs at all time points (**Figure 16 A**). Although frequencies of small intestinal ILC1 and ILC3 converged at E18.5, ILC3 established as the major ILC population over time. In FL, comparable frequencies of ILC1 and ILC3 could be observed in E14.5 and percentages of ILC3 decreased successively with concomitant increase in ILC1 (**Figure 16 A**). The percentage of CD4⁺T-bet⁺ ILC3 increased gradually in the intestine, while CD4⁺T-bet⁻ and CD4⁺T-bet⁺ ILC3 decreased from E14.5 to 4 weeks of age (**Figure 16 B and C**). In line with previous findings (Sawa et al., 2010), NKp46 was not detected on the surface of ILC3 during embryonic development and appeared only in newborn mice, regardless of T-bet expression (**Figure 16 B**). Expression of CCR6 was selectively observed by a minor fraction of intestinal CD4⁺T-bet⁻ ILC3 cells by E14.5 (**Figure 15 F**), while its expression increased until E18.5, when CCR6⁺CD4⁺ ILC3 first appeared (**Figure 16 D**). The frequency of CCR6⁺CD4⁺ ILC3 reduced progressively until virtually absent postnatally where CCR6⁺CD4⁺ became the predominant ILC3 populations (**Figure 16 D**).

Figure 16. Kinetics of ILC3 subsets during ontogeny reveal distinct waves of differentiation. (A) Quantification of ILC1/NK or ILC3 frequencies within T-bet⁺ and/or RORγt⁺ group 1/3 ILCs over time in ontogeny in SI and liver. (B) Representative plots of T-bet ZsGreen or NKp46 and CD4 expression by RORγt⁺ ILC3 isolated from SI at different indicated embryonic and postnatal time-points. (C) Quantification of frequency of designated RORγt⁺ ILC3 subsets over time in ontogeny. (D) Representative graphs of E16.5 and E18.5 and quantification over time of CCR6 and/or CD4 expression within RORγt⁺ ILC3 isolated from SI. Graphs depict data as mean ± SEM, n = 3-12. Data are representative of 2-3 independent experiments.

These data depict that a consistent proportion of CD4⁺Tbet⁺ intestinal ILC3 was present at E14.5 in contrast to 4-week-old mice. To test whether embryonic CD4⁺Tbet⁺ ILC3 might undergo lineage reprogramming already during fetal life, genetically fate-labelled mouse models that express cell-heritable fluorescent dyes in a cell type-specific manner were analyzed. These mice enable help to define relationships between cell types and provide information on lineage origin. To this end, newborn fate map *Rorc(gt)^{cre} x R26R^{eYFP}* (ROR γ t-FM) or *Tbx21^{cre} x R26R^{eYFP}* (T-bet-FM) reporter mice were analyzed as the frequency of CD4⁺Tbet⁺ ILC3 was already strongly diminished at birth. By analyzing ROR γ t-FM mice indeed, a population of ILC3 was found that had switched off ROR γ t expression converting into ex ILC3 as it has also been previously described in adult intestine under the influence of IL-12 (Bernink et al., 2013; Klose et al., 2013; Vonarbourg et al., 2010). Specifically, a population of NK1.1⁺ROR γ t-FM⁺ cells was found within T-bet⁺ROR γ t⁺ ILC1 in both intestine and liver, showing that ex ILC3 are not only generated postnatally, but are already present at birth (Figure 17 A and B). Reciprocally, a population of T-bet-FM⁺ cells among ROR γ t⁺CD4⁺ ILC3 was detected in the intestine of newborn T-bet-FM reporter mice, suggesting a history of T-bet expression among LTi cells (Figure 17 C).

Figure 17. ILC3 plasticity starts prenatally.

Representative gating on T-bet⁺ ROR γ t-protein⁻ ILC1 and identification of NK1.1⁺ROR γ t-fate map (FM)⁺ ex-ILC3 in one day old *Rorc(gt)^{cre/wt} x R26^{eYFP}* newborn mice in SI (A) and liver (B). (C) Representative SI gating on ROR γ t-protein⁺CD4⁺ LTi cells and identification of T-bet-protein⁺T-bet-FM⁺ ILC3 in one day old *Tbx21^{cre/wt} x R26^{eYFP}* newborn mice. Quantification of frequencies and absolute numbers as mean $\pm$ SEM. Data are representative of 2-3 independent experiments (n = 3-6) per time-point.

Altogether, T-bet was expressed by subsets of intestinal ILC3 during embryonic development; explicitly a population of CD4⁺Tbet⁺ ILC3 was identified, which gradually decreased during development suggesting a possible conversion of ILC3 and ILC1 fates, highlighting a previously unappreciated prenatal ILC plasticity.

4.5 T-bet deficiency rescues formation of peripheral LN in ROR γ t-deficient mice

Following up on the finding of T-bet expression in fetal ILC3, the effect of its expression on embryonic ILC3 functions, namely LN formation, was investigated next. To this point, *Tbx21*^{-/-} *x* *Rorc(gt)*^{GFP/wt} mice were analyzed with *Rorc(gt)*^{GFP/wt} reporter mice as controls, demonstrating that LN generation in adult *Tbx21*^{-/-} *x* *Rorc(gt)*^{GFP/wt} mice was largely normal (**Figure 18 A-C**) (Rankin et al., 2013).

Figure 18. T-bet knockout mice have normal development of LN. (A) *In vivo* photos of mesenteric or inguinal lymph nodes (mLN, iLN) of adult *Tbx21*^{-/-} *x* *Rorc(gt)*^{GFP/GFP} mice or after isolation (B). (C) Quantification (n = 12) of frequencies of mice with lymphoid structures compared to control mouse strain in adult animals. PP's, Peyer's patches.

It is well established that T-bet and ROR γ t are co-expressed within adult ILC3 where they cross-regulate each other (Klose et al., 2013; Rankin et al., 2013; Zhong et al., 2016). To examine the influence of both TFs for embryonic ILC3 development, *Rorc(gt)*^{GFP/GFP} *x* *Tbx21*^{-/-} double knockout (DKO) mice were generated. While ROR γ t deficiency in *Rorc(gt)*^{GFP/wt} mice led to dramatic impairment of peripheral LN generation as previously reported (Eberl et al., 2004; Sun et al., 2000), analysis of *Rorc(gt)*^{GFP/GFP} *x* *Tbx21*^{-/-} mice, lacking both T-bet and ROR γ t, resulted in complete rescue of sacral, cervical, axillary and mesenteric LN, whereas emergence of brachial, inguinal and renal LN occurred in around 30-60% and PP (Figure 19 A-C).

Figure 19. T-bet deficiency rescues LN formation in ROR γ t-deficient mice. (A) *In vivo* photos of mesenteric or inguinal lymph nodes (mLN, iLN) of adult animals in indicated mouse strains. (B) Photos of isolated mLN in indicated mouse strains. (C) Quantification (n = 12) of frequencies of mice with lymphoid structures compared to control mouse strain *Rorc(gt)*^{GFP/wt} in adult animals. PP's, Peyer's patches; nd, not detected.

LN architecture and functions in mLN from *Rorc(gt)^{GFP/GFP} × Tbx21^{-/-}* mice were normal, as evaluated by the presence of germinal centres (GCs) that were marked by PNA-binding B cell clusters and surrounded by IgD⁺ naive B cells. GCs exhibited a discrete segregation of dark and light zone, where conventional CD35⁺ follicular dendritic cells were present (**Figure 20 A**). Moreover, flow cytometric analysis of mLN from *Rorc(gt)^{GFP/GFP} × Tbx21^{-/-}* mice identified equal numbers of T follicular helper (Tfh) cells characterized by CXCR5⁺PD1⁺ as well as ICOS and Bcl-6 expression in addition to IgD^{+/lo}CD38⁺Fas⁺GL7⁺ GC B cells when compared to control mice, revealing functional competence of these mLN (**Figure 20 B**).

Figure 20. LN from ROR γ t/T-bet-DKO mice have normal architecture and are functional. (A) Immunofluorescence of mLN in *Rorc(gt)^{GFP/GFP} × Tbx21^{-/-}* mice. These data were acquired by Dr. Carolin Ulbricht. DZ, dark zone; LZ, light zone; SCS; subcapsular sinus. (B) Representative flow cytometric plots of T follicular helper (Tfh) cells (upper row, gated as LD⁺CD45⁺CD3⁺CD4⁺CXCR5⁺PD1⁺) and germinal center (GC) B cells (lower row, gated as LD⁺CD45⁺CD3⁺B220⁺CD19⁺GL7⁺Fas⁺). Histograms show expression of Bcl-6 and ICOS in Tfh cells, control population consists of non-Tfh CD4⁺ T cells; and CD38 as well as IgD expression in GC B cells, control population consists of non-GC B cells. Black line represents *Rorc(gt)^{GFP/wt}* mice, blue line represents *Rorc(gt)^{GFP/GFP} × Tbx21^{-/-}* mice. Quantification of Tfh and GC B cells in the different mouse strains. Frequencies are shown as mean ± SEM, data are representative of 2 independent experiments (n = 3) per time-point. Mann-Whitney U test; ns, not significant.

In order to exclude a possible role for T or B cells in shaping the observed LN phenotype, *Rorc(gt)^{GFP/GFP} × Tbx21^{-/-}* were crossed to *Rag2^{-/-}* mice. Analysis of residual mLN in these mice revealed analogously rescued development in *Rag2^{-/-} × Rorc(gt)^{GFP/GFP} × Tbx21^{-/-}* mice as opposed to *Rag2^{-/-} × Rorc(gt)^{GFP/GFP}* strain (**Figure 21 A and B**).

Figure 21. Re-appearance of LN is independent of RAG. (A) Photos of mesenteric or inguinal lymph nodes (mLN, iLN) of adult animals in indicated mouse strains. **(B)** Quantification (n = 12) of frequencies of mice with lymphoid structures compared to control mouse strain in adult animals. Nd, not detected.

These results indicate that in the presence of ROR γ t, T-bet does not affect embryonic LT_i cell functions; however, in the absence of ROR γ t, T-bet impedes LN formation independently of T and B cells as evidenced by re-appearance of LN in ROR γ t and T-bet double knockout mice.

4.6 Block of ILC1 and ILC3 differentiation in *Rorc(gt)^{GFP/GFP} x Tbx21^{-/-}* mice favors the accumulation of ILCP

To address whether LN formation in *Rorc(gt)^{GFP/GFP} x Tbx21^{-/-}* mice was associated with the presence of an ILC population displaying LT_i cell functions, LinLD⁻CD45⁺CD122⁺ and/or CD127⁺ cells isolated from E18.5 SI of *Rorc(gt)^{GFP/GFP} x Tbx21^{-/-}* (DKO), were compared to *Rorc(gt)^{GFP/GFP}* (RKO) or *Rorc(gt)^{GFP/wt}* (reporter) mice by scRNA-seq. Collective analysis of equalized cell numbers of all three mouse strains revealed overlapping ILC clusters displaying analogous gene expression signatures in RKO and DKO mice as seen in reporter mice, though with clearly different distributions (**Figure 22 A and B**). As expected from ROR γ t deficiency, ILC3 populations were drastically reduced in both RKO and DKO strains. This effect was paralleled by a conspicuous increase of ILC2 in RKO and DKO mice as well as of ILC1/NK in RKO mice, indicating a competitive cross-regulation between the different ILC lineages in the absence of either TF (**Figure 22 A and B**). Due to T-bet deficiency, DKO mice were defective of the ILC1 cluster, while few cells expressing *Eomes* could be still detected as NK cells can still generate in mice lacking T-bet (**Figure 22 C**) (Townsend et al., 2004). Investigating the progenitor populations, a remarkable overrepresentation of the ILCP cluster was observed in DKO mice, while frequencies of CLP remained equal (**Figure 22 A and B**). Gene signatures such as ILC-lineage markers *Id2*, *Ets1*, *Rora*, *Ahr*, *Cxcr6*, *Arg1*, *Il7r* and *Il2rb*, as well as of ILCP-associated genes *Zbtb16*, *Tcf7* and *Tox* showed comparable expression in ILCP from all strains (**Figure 22 D and E**). In addition, α ₄ β ₇ and PD-1 was detected on the surface of DKO ILCP (**Figure 22 F**),

as also seen in ILCP of reporter mice (**Figure 12 B**). Altogether, comparable analysis of all mouse strains by scRNA-seq exposed an accumulation of ILCP in DKO mice that was analyzed in more detail next to reveal potential association to LN re-appearance.

Figure 22. Single-cell RNA sequencing of fetal ILC populations reveal accumulation of ILCP in ROR γ t/T-bet-DKO mice. Viable Lin(CD19, CD3, CD5, F4/80, Fc ϵ R1 α , Gr-1)⁻ CD45⁺ cells expressing IL-7 receptor (CD127) and/or the IL-2 receptor subunit beta (CD122) isolated from the small intestine (SI) of E18.5 *Rorc(gt)^{GFP/GFP}* (RKO) and *Rorc(gt)^{GFP/GFP} x Tbx21^{-/-}* (DKO) embryos were sort-purified by flow cytometry, and a single-cell expression library was generated using 10x Genomics. **(A)** UMAP dimensional reduction projection identifies 8 distinct clusters. **(B)** Quantification of single cells mapping to correspondent clusters (legend see in A) in indicated mouse strains. **(C)** Selected gene expression within clusters and mouse strains. CLP and ILC3 from reporter mice compared to ILCPs of all strains. Color scale represents average expression, dot size visualizes fraction of cells within the cluster expressing the gene. **(D)** Expression of selected ILCP-associated genes in cells from ILCP cluster. **(E)** Expression of selected proteins on UMAP projection analyzed by CITE-Seq. **(F)** Selected protein expression on cells from DKO mice on UMAP plots.

4.7 PLZF^{hi} ILCP from DKO mice are enriched in cells with LTi signatures

Following up on the finding of increased ILCP in DKO mice, their transcriptional profile and differentiation commitment in DKO, RKO and reporter ILCP was further examined by evaluating the enrichment score of ILCP, ILC1, ILC2 or ILC3 gene modules, defined in the unperturbed subsets of reporter mice (**Figure 23 A**). As a result of ROR γ t deficiency, RKO ILCP were enriched

for the ILC1 and ILC2, but not for the ILC3 module (**Figure 23 A**). In this regard, *Tbx21* transcripts were more abundant in ILCP of RKO than in reporter mice (**Figure 23 B**), suggesting that in the absence of ROR γ t counterbalance, expression of T-bet is enforced and skews ILCP maturation towards ILC1 lineage. On the contrary, the additional lack of T-bet in DKO mice caused accumulation of an ILCP population displaying higher enrichment score for ILC3, but not for ILC1 or ILC2 core modules (**Figure 23 A**). Interestingly, DKO ILCP with greater enrichment for ILC3 genes exhibited a lower enrichment score for the ILCP gene module (**Figure 23 C**). Examination of the top 100 genes defining the ILC3 core module in ILCP from DKO mice revealed no or only little expression of typical ILC3 genes, such as *Il1r1*, *Il17re*, *Nrp1* or *Ccr6*, similar to their ILCP counterparts from reporter or RKO strains (**Figure 23 D and E**). In contrast, a fraction of DKO ILCP expressed higher transcripts for a defined set of ILC3 identifying genes as compared to reporter or RKO mice. Besides *Gpr183*, *Batf3* and *Bcl2* (**Figure 23 and F**), DKO ILCP expressed higher levels of transcripts for genes associated with LT α i cell phenotype and functions, namely *Lta*, *Ltb*, *Cxcr5* and *Tnfsf11* (encoding RANKL) (**Figure 23 G**). Of note, while the absence of ROR γ t in both RKO and DKO mice clearly affected expression of ROR γ t-driven signatures evidenced by the absence of ILC3 clusters (**Figure 22 A and B**), residual *Rorc* transcripts could still be observed within the ILCP from RKO and DKO strains (**Figure 23 D**), possibly indicating residual *Rorc* promoter activity.

Figure 23. Gene expressions patterns in ILCP show enhanced LT α i expression patterns in DKO mice. (A) Violin plots for enrichment score of gene modules of top 100 differentially expressed genes from ILCP or ILC1, ILC2, ILC3 cluster, defined in reporter mice, in single cells from ILCP cluster in indicated mouse strains. E18.5 scRNA-seq dataset, legend see in B. Statistical significance was calculated using Wilcoxon test with Bonferroni correction **(B)** Expression levels of *Tbx21* within ILCP of all strains. **(C)** Co-enrichment plots of ILCP versus ILC3 gene modules within single cells of ILCP cluster in mouse strains. **(D)** Gene expression profiles of the top 100 genes differentially expressed in ILC3 of reporter mice within cells from ILCP cluster. **(E-G)** Violin plots depicting expression of selected transcripts in ILCP of the three mouse strains, legend see in B.

In order to validate the data generated by scRNA-seq, additional flow cytometric analysis of LinLD⁺CD127⁺ and/or CD122⁺ cells derived from E18.5 SI and mLN anlagen was performed. Among CXCR6⁺ cells, a GFP⁺ population was detected in DKO and to lower frequencies also in RKO mice indicating *Rorc* promoter activity and translation of the knock-in sequence encoding GFP in these mice. The observed GFP signal was in line with the observed *Rorc* transcripts by scRNA-seq **(Figure 24 A)**. Furthermore, cells expressing LT α i functional molecules such as the

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LT $\alpha_1\beta_2$ heterotrimer, as well as CXCR5 and RANKL was present in SI and mLN at comparable frequencies to reporter mice while strongly reduced in RKO mice (**Figure 24 B, C, E**). Notably, in reporter mice these populations largely comprised of ROR γ t⁺ ILC3. Although generally detected at low frequencies in FL, the frequency of CXCR5⁺RANKL⁺ ILCs was increased in DKO mice, suggesting that skewed ILCP differentiation towards acquisition of LTi signatures in DKO mice seems to occur already in the FL (**Figure 24 D**). In order to address whether CXCR6⁺ cells in DKO mice corresponded to ILCP, expression of PLZF was assessed. Whereas CXCR6⁺ ILCs from reporter mice expressed low levels of PLZF, CXCR6⁺PLZF^{hi} cells were selectively enriched in SI and mLN of DKO mice (**Figure 24 F**), where co-expression of PD-1 was seen likewise (**Figure 24 G**).

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Figure 24. ILCP from DKO mice are enriched in cells with LTi signatures. (A) Representative flow cytometry from E18.5 SI of LinLD⁻CD45⁺ CD127⁺ and/or CD122⁺ cells. **(A)** Quantification CXCR6⁺GFP⁺ cells (n = 7) and **(B)** of CXCR6⁺LT α β ₂⁺ cells (n = 3) in the different mouse strains shown, see legend in D. **(C)** Quantification of CXCR5⁺RANKL⁺ (n=10) in SI or fetal liver (FL) in **(D)**. A-D, Kruskal-Wallis significance and Dunn's correction. **(E)** Representative flow cytometric plot of pooled E18.5 mLN anlagen gated on LinLD⁻CD45⁺CD127⁺ and/or CD122⁺ cells. **(F)** Representative flow cytometric histogram of LinLD⁻CD45⁺CD127⁺ and/or CD122⁺CXCR6⁺ cells; control population (ctrl. pop.) defined as CD127⁻CD122⁻ of reporter mice. **(G)** Representative flow cytometric plot of pooled E18.5 mLN anlagen gated on LinLD⁻CD45⁺CD127⁺ and/or CD122⁺ cells.

To find out whether accumulation of DKO ILCP was due to enhanced proliferation, Ki67 expression was analyzed and revealed that transcripts as well as protein expression were comparable in ILCP from all three strains, thereby excluding this mechanism for increased ILCP in DKO mice (**Figure 25 A-C**).

Figure 25. Equal Ki67 expression within ILCP of reporter, RKO and DKO mouse strains. (A) Violin plot for *Mki67* transcripts among ILCP in all mouse strains, legend see in B. **(B, C)** Quantification and representative flow cytometry of Ki67 expression in LinLD⁻CD45⁺CD127⁺ROR γ t⁺PD1^{hi}PLZF^{hi} cells from E15.5 SI (n = 4-6). Quantification as mean $\pm$ SEM with Kruskal-Wallis significance and Dunn's correction.

Thus, both scRNA-seq and flow cytometry analysis identified an ILCP population expressing CXCR6, PLZF, PD-1 and key LTi molecules that accumulated in FL, SI and mLN anlage of DKO mice. Overall, these results imply that lack of functional ROR γ t prevents the development of fully mature ILC3; however, in the absence of T-bet, differentiation of ILCP with distinct LTi features is enabled.

To complement the *in vivo* data, *in vitro* differentiation assays culturing sorted FL-derived progenitors on stromal OP9 feeder cells were performed. After one week of culture in the presence of stem cell factor (SCF) and IL-7, all ILC lineages could be generated from progenitors obtained from reporter mice (**Figure 26 A, B**). As also seen *ex vivo*, ILC2 differentiation was increased in cultures from RKO and DKO mice, while generation of NK1.1⁺ cells (marking both ILC1 and NK cells) was additionally reduced in DKO as compared to RKO mice (**Figure 26 A**). Importantly, cultures from DKO, but not from RKO, progenitors enabled the generation of a population of RANKL⁺CXCR5⁺ cells, which co-expressed PLZF (**Figure 26 B**).

Figure 26. In vitro OP9 differentiation cultures. In vitro differentiation of E14.5 fetal liver-derived ILC progenitors on OP9 stromal cells after culture for 5-8 days in the presence of SCF and IL-7 and analysis by flow cytometry. **(A)** Representative flow cytometry plots and quantification of LinLD⁻CD45⁺ICOS^{hi}GATA3^{hi} ILC2 and LinLD⁻CD45⁺ICOS^{hi}NK1.1⁺ group 1 ILCs cells in the different mouse strains, see legend in B. **(B)** Representative flow cytometry plots of CXCR5⁺RANKL⁺ cells among LinLD⁻CD45⁺GATA3^{hi} NK1.1⁻ in the different mouse strains. Quantification shown as mean ± SEM, data are representative of 2-3 independent experiments (n = 3-5) per time-point. Kruskal-Wallis significance and Dunn's correction.

4.8 PLZF^{hi} CXCR6⁺ GFP⁺ cells persist in mLN and intestine of adult DKO mice

Having identified an ILCP population with LT_i features in embryos of DKO mice, 4-week-old mice were examined next to investigate whether such a population would persist into adulthood. CXCR6⁺PLZF⁺ and GFP⁺ cells were present in mLN and SI of adult DKO mice (**Figure 27 A-C**). Importantly, the GFP signal resolved GFP^{lo} cells found in both RKO and DKO mice as well as GFP^{hi} ILCs which were absent in SI of RKO but detected at similar frequencies in DKO and reporter mice (**Figure 27 C**). Conversely to RKO mice, DKO GFP⁺ ILCs partially expressed CD4, but not NKp46 (**Figure 27 C**). Likewise, GFP^{hi} ILCs appeared in the SI of *Rag2*^{-/-} DKO but not in *Rag2*^{-/-} RKO mice (**Figure 27 D**).

Figure 27. PLZF^{hi} CXCR6⁺ ILCP persist in LN and intestine of adult DKO mice. (A, B) Flow cytometry representative plots of mLN from 4-week-old reporter and DKO mice. Quantification of frequencies from two to three independent experiments (n = 8-16), mean ± SEM and Mann-Whitney-U testing. (C, D) Representative flow cytometry plots of SI from indicated mouse strains. Quantification of RORγt-GFP^{lo} and RORγt-GFP^{hi} populations. Data depicted as mean ± SEM, Kruskal-Wallis testing with Dunn's multiple correction, n = 4-12.

Bulk RNA-sequencing of NKp46⁺ and of CD4⁺GFP⁺ populations sorted from SI of adult reporter, RKO and DKO mice underlined the observations gained in the E18.5 single-cell approach. DKO CD4⁺GFP⁺ cells expressed abundant transcripts for *Zbtb16*, *Tox* and *Tcf7*, while lacking other ILC3-related genes including *Il1r1* and *Ccr6*. Of note, CD4⁺ GFP⁺ cells from DKO and reporter mice displayed comparable levels of transcripts for central LTi cell molecules, such as *Lta*, *Ltb*, *Tnfsf11* and *Cxcr5* (Figure 28 A). Additionally, *Il22*, but not *Il17a*, transcripts were restored to similar amounts in DKO CD4⁺ GFP⁺ cells as in NKp46⁺ ILC3 from reporter mice (Figure 28 A and B). These data could be confirmed at the protein level by intracellular staining of cytokines after *ex vivo* restimulation, showing that DKO CD4⁺GFP⁺ cells showcased IL-22, but not IL-17A protein, in line with the transcriptional data (Figure 28 C). Since IL-22 is crucial for induction of AMP it has an essential role in barrier homeostasis within the intestinal epithelium. To test whether partial restoration of IL-22 expression in DKO ILCs could rescue the expression of transcripts encoding for Reg3γ and Reg3β proteins in the intestinal epithelium, *Reg3g* and *Reg3b* transcripts were measured in the SI epithelial cells from DKO, RKO and reporter mice. Notably, while RKO mice displayed dramatic reduction in *Reg3g* and *Reg3b* transcripts, DKO had comparable levels to reporter mice, indicating that partial IL-22 restoration observed in DKO ILCs is sufficient to restore important features of the intestinal barrier (Figure 28 D).

Figure 28. ROR γ t/T-bet-deficient ILCP produce IL-22 and promote intestinal barrier functions in adult mice. (A) Heatmap of bulk RNA-seq of ROR γ t-GFP⁺ population sort-purified according to NKp46 or CD4 expression isolated from SI of 4-week-old mice. Selected gene transcripts are shown as Z-score. (B) Normalized expression values of *I/22* transcripts from bulk RNA-seq dataset and (C) IL-17A and IL-22 protein expression in SI of DKO mice determined by flow cytometry after 4h *ex vivo* restimulation using PMA/iono/IL-23. Quantification of frequencies from three independent experiments (n = 11-16), mean $\pm$ SEM and Kruskal-Wallis testing with Dunn's multiple correction. (D) Expression of *Reg3g* and *Reg3b* in intestinal epithelial compartment determined by quantitative PCR. Values are normalized to housekeeping genes *Actb*, *Hprt* and *Gapdh*. Each symbol represents an individual mouse. Data show mean $\pm$ SEM, Kruskal-Wallis testing with Dunn's multiple correction, n = 6, see legend in C.

Collectively, these data demonstrate that in the absence of T-bet and ROR γ t, PLZF^{hi} ILCP with LT α i signatures and residual *Rorc* promoter activity develop during embryonic life and persist into adulthood. To supplement the results from *in vitro* differentiation assays and to determine whether this effect was cell intrinsic *in vivo*, adoptive transfer experiments were conducted. To this end, irradiated CD45.1⁺CD90.2⁺ *Rag2*^{-/-}*Il2rg*^{-/-} recipient mice received progenitors derived from FL of CD90.1⁺ strains from reporter, RKO or DKO all expressing CD45.2. In order to deplete residual radio-resistant host ILCs (Gasteiger et al., 2015; Hanash et al., 2012; Robinette et al., 2017), host mice were treated bi-weekly with anti-CD90.2 antibody (schematic overview on experimental setup see Figure 8, page 25 in material and methods section). Four weeks after reconstitution, DKO progenitors efficiently engendered a population of GFP^{hi} cells at similar frequencies as observed in reporter mice, equivalent to the phenotype observed *ex vivo* in adult mice (Figure 29).

Figure 29. GFP⁺ ILCP are generated in *in vivo* adoptive transfer experimental setup. Representative flow cytometry in SI 4 weeks after adoptive transfer and quantification of frequencies of ROR γ t-GFP^{hi} ILCPs as mean $\pm$ SEM (n = 2-9), Kruskal-Wallis significance with Dunn's correction. Data are representative of 2-3 experiments.

Together, these results demonstrate that deficiency of T-bet and ROR γ t facilitates the generation and accumulation of embryonic PLZF^{hi} ILCP, which can persist into adulthood. On the one hand, the absence of T-bet during ILCP differentiation enables expression of molecules crucial for LTi function thereby promoting LN formation, during embryonic development, while on the other hand, IL-22 production restores barrier functions during adulthood in DKO mice.

4.9 LN development and accumulation of PLZF^{hi} ILCP in DKO mice is promoted by ROR α

Since residual transcriptional activity at the *Rorc* locus was observed and GFP^{lo/hi} expression was detected in both RKO and DKO mice, phenotypes were validated in alternative models of ROR γ t deficiency to exclude that LN generation and accumulation of ILCP in KO mice might be due to residual expression of ROR γ t in DKO mice. *Rorc(gt)^{GFP/GFP}* (RKO) mice have been genetically modified by substitution of *Rorc* exon 1 γ t with a DNA sequence encoding GFP inserted directly after the transcriptional start site (TSS) sequence ATG, replacing exon 1 γ t (**Figure 30**) (Eberl et al., 2004). Hence, heterozygously bred mice report expression of ROR γ t by GFP, while in homozygously bred mice both alleles of *Rorc* exon 1 γ t are replaced by GFP, resulting in loss of ROR γ t (knockout). To rule out that the observed phenotype is caused by functional activity of a potential residual protein transcribed from exon 3-11, *Rorc(gt)^{Δex4}* mice, which carry a conventional deletion in exon 4 of the *Rorc* locus (Sun et al., 2000) were crossed to *Tbx21*^{-/-} mice. Lack of exon 4 leads to disruption of the DNA-binding domain (DBD) of ROR γ t (**Figure 30**). Moreover, *Rorc(gt)^{fl/fl}* mice, in which *Rorc* exons 3 to 6 are floxed and cre recombinase activity deletes not only the DBD but also hinge and ligand-binding domain (LBD) regions of ROR γ t (**Figure 30**) (Choi et al., 2016), were crossed to *Tbx21*^{fl/fl} and *Il7*^{cre/wt} mouse lines, enabling conditional deletion of T-bet as well as ROR γ t exon 3-6 in IL-7R⁺ lymphocytes, including ILC progenitors from the CLP stage onwards.

Figure 30. Schematic overview of *Rorc* locus and *Rorc* deletions in mouse models. In *Rorc(gt)^{GFP/GFP}* mice GFP is inserted directly after the transcriptional start site ATG of exon 1 γ t while *Rorc(gt)^{Δex4}* have a conventional deletion in exon 4 that encodes for the DNA-binding domain (DBD). In *Rorc(gt)^{fl/fl}* mice exon 3-6 are surrounded by floxed regions and under cre-recombinase activity delete DBD, hinge and ligand-binding domain (LBD) of ROR γ t.

Notably, thymic disruption of T cell development typically associated to ROR γ t deficiency (Guo et al., 2016; He et al., 2000; Sun et al., 2000) was observed in both ROR γ t single-deficient mouse models (*Rorc(gt)^{GFP/GFP}* and *Rorc(gt)^{Δex4}* mice) and could not be rescued in ROR γ t/T-bet double-deficient (*Rorc(gt)^{GFP/GFP} x Tbx21^{-/-}* and *Rorc(gt)^{Δex4} x Tbx21^{-/-}*) mice. Likewise, thymic compartments were similarly impaired in *Il7^{cre/wt} x Rorc(gt)^{fl/fl} x Tbx21^{fl/fl}* mice, altogether corroborating the absence of functional ROR γ t activity (Figure 31).

Figure 31. Thymic CD4⁺CD8⁺ compartments are not restored in ROR γ t/T-bet double-deficient mouse models. Flow cytometry representative plots of thymi from 4-week-old mice of indicated mouse strains.

Markedly, both additional models of concomitant ROR γ t and T-bet deletion phenocopied *Rorc(gt)^{GFP/GFP} x Tbx21^{-/-}* mice, resulting in presence of LN which were absent in the ROR γ t single-deficient counterparts (*Rorc(gt)^{lex4}* and *Il7^{cre/wt} x Rorc(gt)^{fl/fl}* mice) (**Figure 32 A and B**). In support of this phenotype, PLZF^{hi} ILCP accumulated in mLN of both additional mouse strains lacking ROR γ t and T-bet (**Figure 32 C and D**), matching the observations in *Rorc(gt)^{GFP/GFP} x Tbx21^{-/-}* DKO versus *Rorc(gt)^{GFP/wt}* reporter mice.

Figure 32. LN formation and accumulation of PLZF⁺ ILCP is independent of DNA- and ligand-binding domains of ROR γ t in the absence of T-bet. (A, B) Quantification (n = 12) of frequencies of mice with lymphoid structures compared to control mouse strain in adult animals. PP's, Peyer's patches; nd, not detected. (C, D) Flow cytometry representative plots of mLN from 4-week-old mice of indicated mouse strains. Quantification of frequencies from two to three independent experiments as mean $\pm$ SEM (n = 6-8), Mann-Whitney-U testing.

Together, these results demonstrate that, in the absence of T-bet, LN formation and accumulation of PLZF^{hi} ILCP with LTi signatures occur irrespective of ROR γ t TSS ATG, DBD, hinge

Results

and LBD regions, indicating that acquisition of LT α functions, but not full ILC3 maturation or thymic T cell development, can happen independently of ROR γ t.

Finally, to assess whether other transcription factors promote acquisition of LT α profile and LN formation in the absence of ROR γ t and T-bet, the importance of ROR α (closely related to ROR γ t) was analysed, which has been shown to cooperatively regulate Th17 genes conjointly with ROR γ t (Castro et al., 2017; Sundrud and Rao, 2008; Yang et al., 2008a). In the E18.5 scRNA-seq dataset, *Rora* was found to be highly expressed in ILCP from all mouse strains (Figure 33 A). To test whether ROR α could substitute absence of ROR γ t and account for generation of ILCP displaying LT α activity *Il7^{cre/wt} x Rorc(gt)^{fl/fl} x Tbx21^{fl/fl}* mice were crossed to *Rora^{fl/fl}* mice and presence of LN was assessed. Strikingly, *Il7^{cre/wt} x Rorc(gt)^{fl/fl} x Tbx21^{fl/fl} x Rora^{fl/fl}* mice showcased complete absence of all LN (Figure 33 B). Along this line, intestinal PLZF^{hi} ILCP did not generate in triple-floxed mice compared to double-floxed animals (Figure 33 C).

Figure 33. ROR α promotes LN development and PLZF⁺ ILCP accumulation in DKO mice. (A) Violin plot of *Rora* expression within ILCP of designated mouse strains from E18.5 scRNA-seq dataset. (B) Quantification (n = 12) of frequencies of mice with lymphoid structures compared to control mouse strain in adult animals. PP's, Peyer's patches; nd, not detected. (C) Flow cytometry representative plots of SI from 4-week-old mice of indicated mouse strains. Quantification of frequencies from two to three independent experiments as mean $\pm$ SEM (n = 8-9), Kruskal-Wallis testing with Dunn's multiple correction.

Altogether, the present study reveals a complex interplay between T-bet, ROR γ t and ROR α in regulating fetal ILCP differentiation and unravel a previously unrecognized role for T-bet and ROR α in LN organogenesis during embryonic life.

5 Discussion

The fetal intestine holds mature ILC subsets and immature progenitors

ILCs are among the first lymphocytes to appear during ontogeny to seed their respective organs and tissues even before cells of the acquired immune system are generated. Nevertheless, apart from broad description in the intestine of adult animals also at single-cell level ([Gury-BenAri et al., 2016](#); [Hernández et al., 2018](#)), their transcriptomic profile in the fetus has not been intensively studied to date. To identify not only the embryonic transcriptional profile of ILCs but also to characterize their phenotype in parallel, proteogenomic single-cell RNA sequencing was performed that allows to accurately understand fetal ILC diversity. Since the development and homeostasis of ILCs depends on stromal IL-7, CD127 has evolved as a defining marker for all ILCs. However, CD127 expression varies throughout ILC subsets and tissues. In the current study, all members of the ILC family were effectively sorted by using CD127 and additionally CD122 as ILC surface markers, furthermore revealing progenitor populations that could be defined as CLPs and ILCPs within the isolated tissue. Intriguingly, accumulating evidence suggests that local pools of tissue-resident ILCs originate from circulating naive progenitor cells that mature within the tissue ([Lim et al., 2017](#); [Nagasawa et al., 2019](#); [Oherle et al., 2020](#); [Zeis et al., 2020](#)). On this matter, Locksley and colleagues have previously described a population of Arg1⁺ ILCP in fetal intestine of Arg1 reporter mice ([Bando et al., 2015](#)), probably largely overlapping with the ILCP detected in this work, which also expressed *Arg1*. Furthermore, CLP and ILCP populations have also been recently reported in peripheral LN anlagen at E13.5 and E14.5 ([Simic et al., 2020](#)), suggesting that FL CLP and ILCP might seed these sites to mature locally. Altogether, these results highlight the embryonic intestine as an active site of differentiation *in situ* during fetal development.

Interestingly, while ILC2 bifurcated at an early stage, trajectory analysis revealed a common lineage development of ILC3 and ILC1/NK. Similar observations were reported in BM-derived progenitors where ILC2 rapidly commit to lineage while putative ILC1, ILC3 and NK progenitors display heterogeneous TF expression with many more transitional states ([Walker et al., 2019](#)). The current study underlines this concept and similarly suggests a greater common transcriptional regulation of ILC3/ILC1 lineages in embryonic ILC differentiation. While ILCP differentiation was highly influenced in mice deficient for ROR γ t and/or T-bet, ILC2 differentiation remained unaffected in the used KO models, pinpointing towards a lineage differentiation program different to ILC3/ILC1 lineages.

Fetal ILC3 are heterogeneous and display T-bet expression

Embryonic LT_i cells were described and considered a rather homogenous population with the central function to organize LN formation. In mature mice, it is well established that ROR γ t and

T-bet are co-expressed within ILC3 where they cross-regulate each other. Remarkably, a certain heterogeneity within the transcriptome of E18.5 intestinal ILC3 was detected in the present work. Besides LT_i cells, the emergence of an unappreciated T-bet⁺ ILC3 subset was observed. Using T-bet ZsGreen reporter mice, the ILC compartments were dissected in a kinetic fashion in the developing intestine and a T-bet⁺CD4⁺ population was found as early as E14.5 displaying some phenotypic similarities with classical LT_i cells. While the current data confirm the prenatal absence of ILC3 expressing NKp46 protein, T-bet transcripts and protein expression starts already as early as E14.5 during embryonic development. The quantitative expression of the two lineage-defining TFs ROR γ t and T-bet may alter their reciprocal balance, influencing phenotype and plasticity of ILC subsets. Indeed, T-bet, GATA3 and ROR γ t haplo-insufficiencies have been reported to affect marker expression and functions (Klein Wolterink et al., 2013; Rankin et al., 2016; Walker et al., 2019; Zhong et al., 2016). Contrary to the prevailing view, the data obtained here indicate that, acquisition of T-bet and generation of plastic transcriptional states by loss of ROR γ t or T-bet, as investigated using ROR γ t-FM and T-bet-FM reporter mice, is not a phenomenon linked to exposure to inflammatory signals postnatally, but can occur already prenatally. Together, these data indicate a prenatal divergence of group 3 ILC subsets with imprinted transcriptional programs, independent of colonization of the intestine with commensal microbiota. The observed transcriptional states in the fetal gut might instead result from other environmental cues such as for instance cellular interactions or local cytokine milieus in the developing tissue. As described in the introduction **section 1.3.3**, environmental signals influencing ROR γ t expression in fetal LT_i cells have been described, such as maternal-derived RA, indicating a direct regulation by localized metabolites (Van De Pavert et al., 2014). It has also been shown in ILC3 of adult mice (**see also section 1.3.2**), that T-bet can be induced by Notch signaling to generate NKp46⁺ subsets (Rankin et al., 2013; Verrier et al., 2016). Such local availabilities of functional molecules or receptors in the evolving intestinal tissue could represent plausible factors influencing TF expression and/or their balance prenatally.

T-bet has furthermore been shown to bind and activate numerous genes like *Cxcr3*, *Ccr5*, *Itgal* and *Icam1*, thereby controlling T cell transcriptional programs and determining lymphocyte homing (Jenner et al., 2009; Lord et al., 2005; Matsuda et al., 2007). A similar influence of T-bet on regulation of chemokine receptor expression in ILC3 might actively control migratory features to sites other than LN anlagen suggesting that heterogenic ILC3 might locate to specific environments. Interestingly, reduced expression of CXCR5, CCR6 and integrin $\alpha_4\beta_7$ was observed in ILC3 that co-expressed T-bet (**Figure 15 F and G**), potentially affecting their homing properties to specific niches.

Notably, further heterogeneity besides type 1-like ILC3 was observed, with one cluster displaying enhanced transcriptional circuits associated to MHCII molecules. These ILC3 emerged in the

absence of CD4⁺ T cells that will only generate after birth. In adult mice, selective ablation of MHCII on ILC3 impaired adaptive T and B cell responses, weakened tolerance to commensals and induced intestinal inflammation (Von Burg et al., 2014; Hepworth et al., 2013, 2015). How this relates to embryonic MHCII-expressing ILC3 and what role tolerance versus immunity plays at this phase in ontogeny remains to be seen. With regards to ILC3-mediated tolerance unrelated to microbiota, embryonic LT_i cells in the thymus were shown to provide RANKL stimulation to medullary thymic epithelial cells (mTECs) to induce expression of autoimmune regulator (Aire) (Roberts et al., 2012). Along this line, Aire⁺ ILC3 with potent antigen-presenting features were found in peripheral LN of adult mice, where those cells could be rarely detected at birth, numbers peaked at the time of weaning and declined thereafter (Yamano et al., 2019). In this context, it will be interesting to clarify whether MHCII⁺ ILC3 with antigen-presenting potential are involved in establishment of early T cell tolerance in the developing intestinal environment prenatally.

Remarkably, MHCII expression by ILC3 was shown to be regulated by the pIV promoter elements of the master TF for MHCII transactivator or CIITA in an IFN γ -dependent manner (Hepworth et al., 2015). Whether IFN γ could serve as a polarizing signal determining the heterogeneity towards MHCII-expressing ILC3 in the embryonic intestine is an interesting question that remains to be addressed in future studies.

Specified ILC3 subsets might indicate a layered ontogeny

Multiple studies over the past years have revealed that diverse lymphoid subsets are exclusively produced during the embryonic to neonatal period such as B-1a B cells locating to peritoneal and pleural cavities or certain subsets of $\gamma\delta$ T cells residing in gut and skin (Böiers et al., 2013; Kobayashi et al., 2014; Yoshimoto et al., 2011, 2012). In this regard, it is progressively becoming clearer from previous mouse studies that ILCs arise from distinct waves during development, a term called layered ontogeny (Oherle et al., 2020; Schneider et al., 2019). Interestingly, in the present study, it was observed that early CD4⁺ LT_i cells were CCR6⁻ and that CCR6⁺ CD4⁻ ILC3 started to establish only between E16.5 and E18.5, suggesting diverse waves of ILC3 differentiation. Considering that, during embryonic development distinct waves of hematopoietic progenitors emerge prior to the generation of HSCs, giving rise to a series of transient progenitors, it is conceivable that ILC3, especially early LT_i cells, might in part originate from different layers to fulfill the immediate needs of the developing embryo. The first wave is initiated at E7 from endothelial cells that undergo Endothelial to Hematopoietic cell Transition (EHT), localizing to the yolk-sac and generating progenitors with potential to give rise to primitive erythrocytes, macrophages and megakaryocytes (Moore and Metcalf, 1970; Palis et al., 1999; Tober et al., 2007). A second wave with broader myeloid potential emerging after establishment of circulation locating to the blood vessel of the yolk sac from E8, to generate erythro-myeloid progenitors (EMPs) (McGrath et al., 2011, 2015b). Only the third final wave, produces cells with

lymphoid potential (LMPPs, lymphoid- and myeloid-primed progenitors) around E10 (Böiers et al., 2013; Ohmura et al., 1999, 2001). At this timepoint, the first HSC are detected within in the aorta-gonad-mesonephros (AGM) region as well as in vitelline and umbilical vessels as extraembryonic sites (De Bruijn et al., 2000; Gordon-Keylock et al., 2013; Kumaravelu et al., 2002; Müller et al., 1994). Later in ontogeny, HSCs mobilize into FL before emigrating into the BM just before birth. By analyzing several mouse lines with time-specific cre expression, van de Pavert et al. describe a detailed view of ILC3 ontogeny (Simic et al., 2020) and suggest that hemogenic progenitors appear from the third wave of hematopoiesis explicitly in early embryonic hemogenic endothelium, giving rise to proliferating precursor populations in FL. They furthermore propose that embryonic LTi cells are replaced by HSC-derived LTi cells in adults. In another study using *Vav^{cre} x Gata3^{fl/fl}* mice it was shown that ILC1/2/3 do not develop where *Vav^{cre}* is active at the HSC stage (Yagi et al., 2014). Surprisingly, *Vav^{cre} x Gata3^{fl/fl}* mice do not generate CD127⁺α4β7⁺ ILCP in adult BM; however such progenitors could be detected in E15.5 FL indicating that these might derive from an early wave before cre-excision. Intriguingly, *Vav^{cre} x Gata3^{fl/fl}* mice harbor a small population of CD4⁺ LTi cells in adult SI, possibly originating from the embryonic progenitors, but do not develop LN (Yagi et al., 2014). Why these mice fail to generate LN is not clarified. It is thinkable, that quantity of LTi cells might be crucial but numbers of embryonic LTi cells were not analyzed in their study.

Whether ILCs originating from these diverse waves of differentiation might differ in their functions, or whether they just need to be localized to specific sites in time and space to accomplish complex developmental phases of tissue or organ development remains to be clarified in future studies. Upcoming technical advances in lineage tracing together with in-depth single cell transcriptional and epigenetic technologies will help to discover new insights into how sequential layers during ontogeny contribute to different lineage progeny and decipher their functional heterogeneity.

RORγt counteracts T-bet during fetal ILC differentiation

With the intention to delineate the role of T-bet in ILC3 lineage specification during embryogenesis, it was found that lack of T-bet restored the development of peripheral LN, typically absent in RORγt-deficient mice. This phenotype was recapitulated in three independent mouse models with different *Rorc* locus disruptions, pinpointing a redundant role for DNA- and LBD-domains as well as hinge region of RORγt for LN formation in the absence of T-bet. Notably, LN formation in all double knockout strains was associated with the appearance and accumulation of an ILCP population expressing PLZF that, due to absence of RORγt and T-bet, is likely not able to differentiate towards mature ILC lineages. Accumulation of PLZF^{hi} ILCP was not accompanied with increased proliferation but might be linked to increased survival, as suggested by elevated expression of the anti-apoptotic molecule *Bcl2*, whose transcripts were enriched among DKO

ILCP (**Figure 23 F**). It was previously shown in *Zbtb16*^{GFPcre} FM mice that PLZF transiently marks a FL and BM ILCP, which has lost LT_i potential, while mature ILCs are largely negative for PLZF (Constantinides et al., 2014). The data here confirm high enrichment of PLZF expression in ILCP from embryonic intestine and mLN anlage but demonstrate that *Zbtb16* expression is also present, albeit at a lower level, in fetal ILCs especially within ILC3/LT_i cells, in line with a recent report by McKenzie et al. (Walker et al., 2019). Using scRNA-seq this study furthermore describes a *Cxcr5*-expressing ILCP in adult BM, and together with data obtained in another study using bulk RNA-seq, they propose CXCR5 as a useful marker to identify ILCP (Harly et al., 2018; Walker et al., 2019). Besides *Cxcr5* transcripts and protein, in the present study expression of LT_i functional molecules such as RANKL and LT $\alpha_1\beta_2$ heterotrimer was detected within embryonic tissue-derived ILCP, preceding expression of *Rorc* that subsequently allows maturation to full LT_i cell phenotype. These data suggest that ROR γ t is important to drive full differentiation of the ILCP towards the ILC3 lineage but seems redundant for the acquisition of central LT_i molecules and related functions, such as LN development. A model of mixed lineage priming (Ishizuka et al., 2016; Walker et al., 2019) with transient upregulation of multiple master TFs has been proposed in differentiating ILCP isolated from E15.5 FL or adult BM, a process also observed here in tissue-derived ILCP. However, upregulation of LT_i signatures within these progenitors only manifested in the absence of T-bet and did not emerge in ROR γ t single deficient mice, where ILCP preferentially differentiate towards ILC1 (and ILC2). Thus, this indicates that ROR γ t has a central role in counteracting T-bet-driven transcriptional programs highlighting the importance of a balanced expression of these two TFs in the regulation of ILC3/ILC1 lineage maturation not only postnatally, but also prenatally.

ROR γ t-independent restoration of IL-22 in the absence of T-bet

ILC3 do not only play a crucial role in fetal development of LN, but also in sustaining intestinal homeostasis postnatally by their production of IL-22. In the absence of ROR γ t and T-bet, PLZF^{hi} ILCP persisted in adult mLN and intestine, and promoted epithelial barrier functions via restored IL-22 production. While the role of ROR α in restoring IL-22 from ILC3 in the absence of ROR γ t and T-bet was not directly ascertained in this study, this has been recently shown in a study accepted for publication (Fiancette et al., 2021, personal communication). Moreover, additional TF might contribute to this effect. For example, the TF Ahr has been shown to be critical for IL-22 production by ILC3 (Kiss et al., 2011; Qiu et al., 2012) but also GATA3 binds to the *Il22* promoter to regulate production within ILC3 (Zhong et al., 2016). Hence, various other factors, also found here to be expressed in ILCP, may compensate for the lack of ROR γ t to foster the production of IL-22. Interestingly, analysis of patients carrying bi-allelic *Rorc* loss-of-function mutations revealed absence of IL-17-producing lymphocytes but residual IL-22 producers suggesting similar compensatory pathways in humans (Okada et al., 2015).

ROR γ t and ROR α act to limit T-bet-driven programs in ILCP

Finally, in regards to which other TF might compensate and promote expression of LTi molecules, ROR α was reported to synergistically regulate Th17 genes together with ROR γ t and has furthermore been described to stably maintain ILC3 lineage (Castro et al., 2017; Lo et al., 2016, 2019; Sundrud and Rao, 2008; Yang et al., 2008b). New data show that ROR α binds to an enhancer element within the *Rorc* locus reinforcing ROR γ t transcriptional programs and crucially stabilizing Th17 lineage maintenance (Lee et al., 2020). Likewise, ROR α is suggested to essentially preserve ILC3 fate during *S. typhimurium* infection and in its absence ILC3 display enhanced core ILC1 genes proposing conversion into ex ILC3 (Lo et al., 2019). While ROR α expression is a common feature of all ILC subsets (Halim et al., 2012; Wong et al., 2012), an additional study also predicted ROR α to repress ILC1/NK gene expression under homeostatic conditions (Pokrovskii et al., 2019). This led to the assumption that ROR α could potentially compensate for the loss of ROR γ t in T-bet-deficient ILCP and endorse expression of LTi cell-associated transcripts. Indeed, its additional deletion in *IL7^{cre/wt} x Rorc(gt)^{f/f} x Tbx21^{f/f} x Rora^{f/f}* mice abolished LN development as well as accumulation of PLZF^{hi} ILCP. Collectively, these findings demonstrate a new role for ROR γ t in counteracting T-bet driven differentiation of embryonic ILCP and reveal ROR α as a crucial regulator of ILCP differentiation and early acquisition of LTi signatures. A strict balance of ROR γ t and T-bet in differentiating ILCP governs the ILC3/ILC1 equilibrium, while ROR α sustains PLZF^{hi} ILCP accumulation and functions before and after birth.

Altogether, the results provided in this study define a novel role for T-bet during fetal ILC3 differentiation and LN generation and unveil a previously unrecognized embryonic ILC3 heterogeneity complementing and advancing the knowledge of transcription factor networks yet described in adult ILC lineages.

5.1 Concluding remarks and open questions

Collectively, this work provides new insights into transcriptional regulations within embryonic ILC and identify TF networks crucial for formation of LN. The key findings are summarized below.

- Single-cell sequencing of fetal intestinal cells identifies mature ILCs and progenitors
- T-bet⁺ ILC3 are already present in the E14.5 intestine, liver and mLN anlage
- T-bet deficiency rescues LN organogenesis in ROR γ t-deficient mice and is associated with accumulation of a PLZF^{hi} ILCP population with LTi functions
- PLZF^{hi} ILCP persist in the adult mLN and intestine and promote epithelial barrier functions via restored IL-22 production

- LN development and accumulation of PLZF^{hi} ILCP with LT_i functions in mice deficient for ROR γ t and T-bet is dependent on ROR α

In view of these novel findings, several open questions remain and might direct potential future subjects of investigation, for example:

Which signals drive early acquisition of T-bet during fetal ILC differentiation (IL-12, IFN γ , Notch)? Where do CLPs and ILCPs localize in the developing intestine? Is there a special niche with distinct environmental settings and special cellular components? Future improvements of techniques such as spatial transcriptomics will help to decipher this issue.

The understanding of the programmed development of secondary lymphoid organs has progressively increased over the last decade and the identified LT_i cells interacting with stromal cells as key players herein. These processes are orchestrated by a variety of chemokines, cytokines and cell-cell contacts; however, the exact mechanisms and signals needed for development of the various distinct lymphoid structures are not clarified in detail at current. In light of the re-occurrence of most but not all LN in ROR γ t/T-bet-DKO mice, what exact role do ILCP with LT_i activity play in the generation of specific LN? Could separate waves of ILC3 differentiation colonize individual LN anlagen? May different ILC3 subsets home to specific sites of LN formation?

Human ILC poiesis is less well characterized and most studies use CD34⁺ CLP-like hematopoietic progenitor cells which can be further delineated by a defined a set of surface markers. Committed ILC3 precursors have been described in human tonsil and intestinal lamina propria and a subset of IL-17-producing fetal LT_i cells was also identified. Are intermediate stages displaying expression of PLZF and LT_i genes also present in humans?

5.2 Graphical summary

Figure 34. Schematic overview on transcription factor networks regulation during ILC differentiation. Differentiation of common lymphoid progenitors (CLP) towards innate lymphoid cell progenitors (ILCP) is marked by upregulation of transcription factors ID2 and PLZF. ROR α promotes expression of lymphoid tissue inducer (LTi) cell activity-associated molecules such as CXCR5, RANKL as well as the heterotrimer LT α 1 β 2 in differentiating ILCP. At this stage, a phase of multilineage priming with upregulation of various lineage-defining transcription factors eventually determines cell progeny fate. The two orphan nuclear receptor transcription factors ROR γ t and ROR α synergistically regulate gene expression, whereas, expression of T-bet counteracts ROR α / γ t-driven signatures and induces a type 1-associated transcriptional program that antagonizes LTi-related gene transcription. Thus, the absence of T-bet skews the differentiation fate of fetal ILCs and promotes accumulation of PLZF⁺ ILCP expressing central LTi molecules. In mature ILC3, ROR γ t is crucial in counteracting the suppressive effects of T-bet.

6 References

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7 Abbreviations

Ahr. Aryl Hydrocarbon Receptor

AMP. Anti-Microbial Peptides

BAC. Bacterial Artificial Chromosome

BM. Bone Marrow

CD. Crohn's Disease

CLP. Common Lymphoid Progenitor

CP. Cryptopatches

DAMP. Damage-Associated Molecular Pattern

DKO. Double Knockout (ROR γ t and T-bet, *Rorc(gt)^{GFP/GFP} x Tbx21^{-/-}*)

DN. "double-negative"

DZ. Dark Zone

FL. Fetal Liver

GFP. Green Fluorescent Protein

GC. Germinal Center

HSC. Hematopoietic Stem Cell

IBD. Inflammatory Bowel Disease

IFN. Interferon

ILC. Innate Lymphoid Cell

ILCP. Innate Lymphoid Cell Progenitor

IL. Interleukin

ILF. Isolated Lymphoid Follicle

LN. Lymph Node

LT. Lymphotoxin

LTi. Lymphoid Tissue inducer

LTin. Lymphoid Tissue initiator

LTo. Lymphoid Tissue Organizer

LZ. Light Zone

M. Microfold (M cells)

MHCII. Major Histocompatibility Complex class II

mLN. Mesenteric Lymph Node

NCR. Natural Cytotoxicity Receptor

NK. Natural Killer

PAMP. Pathogen-Associated Molecular Pattern

PP. Peyer's Patches

RA. Retinoic Acid

Reporter. ROR γ t reporter (*Rorc(gt)^{GFP/wt}*)

RKO. ROR γ t Knockout (*Rorc(gt)^{GFP/GFP}*)

RNA. Ribonucleic Acid

ROR. Retinoic acid receptor-related Orphan Receptor

scRNA-seq. Single-Cell RNA Sequencing

SCS. Subcapsular Sinus

SI. Small Intestine

TF. Transcription Factor

Th. T helper

Tfh. T follicular helper

UC. Ulcerative Colitis

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9 Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die vorliegende Dissertation selbstständig gemäß guter wissenschaftlicher Praxis angefertigt und keine anderen als die angegebenen Hilfsmittel verwendet zu haben.

Weiterhin erkläre ich hiermit, dass die Dissertation oder Teile davon nicht bereits bei einer anderen wissenschaftlichen Einrichtung eingereicht, angenommen oder abgelehnt wurden und auch kein anderwärtiger Doktorgrad erworben wurde.

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